EXHIBIT 1

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United States Patent [19]

Moss et al.

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[54] **PROKARYOTIC EXPRESSION IN EUKARYOTIC CELLS**

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[73] Assignee: **The Research Foundation of State University of New York**, Buffalo, N.Y.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,126,251.

[21] Appl. No.: **187,119**

[22] Filed: **Jan. 26, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 648,971, Jan. 31, 1991, abandoned, which is a continuation-in-part of Ser. No. 582,489, Sep. 14, 1990, abandoned, which is a continuation-in-part of Ser. No. 905,253, Sep. 8, 1986, abandoned.

[51] **Int. Cl.**[6] **C12N 5/10**; C12N 15/09; C12N 7/01

[52] **U.S. Cl.** **435/69.1**; 435/69.3; 435/172.3; 435/193; 435/207; 435/235.1; 435/240.2; 435/240.4

[58] **Field of Search** 435/69.1, 172.3, 435/240.2, 320.1, 69.3, 193, 207, 235.1, 240.4

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,126,251 6/1992 Moss et al. 435/69.1

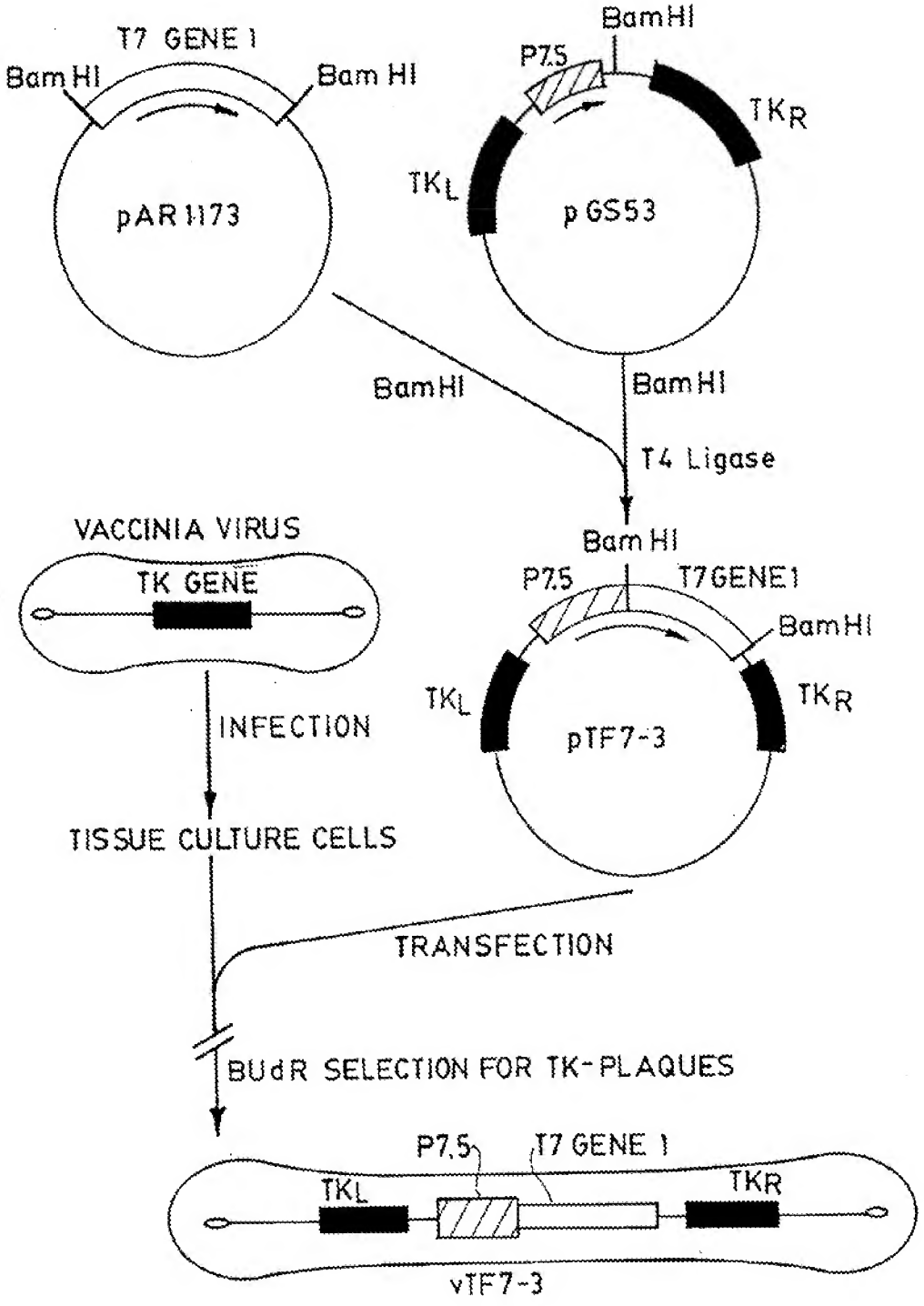
Primary Examiner—Mindy Fleisher
Assistant Examiner—Philip W. Carter
Attorney, Agent, or Firm—Nixon, Hargrave, Devans & Doyle

[57] **ABSTRACT**

A transient expression system is disclosed that utilizes bacteriophage RNA polymerase in the presence of a DNA-based cytoplasmic virus to facilitate expression of a foreign gene in the cytoplasm of a eukaryotic cell.

A method of expressing a foreign gene in the cytoplasm of a eukaryotic cell is also disclosed which comprises incorporating into the cytoplasm a DNA-based cytoplasmic virus, a suitable carrier comprising a gene for an RNA polymerase which gene is foreign to the carrier and to the cells, and a suitable carrier comprising a functional, cistron including a foreign gene flanked by a promotor sequence which is recognized by the RNA polymerase.

43 Claims, 6 Drawing Sheets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INFECTION
TISSUE CULTURE CELLS
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TK$_L$
TK$_R$
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FIG. 1

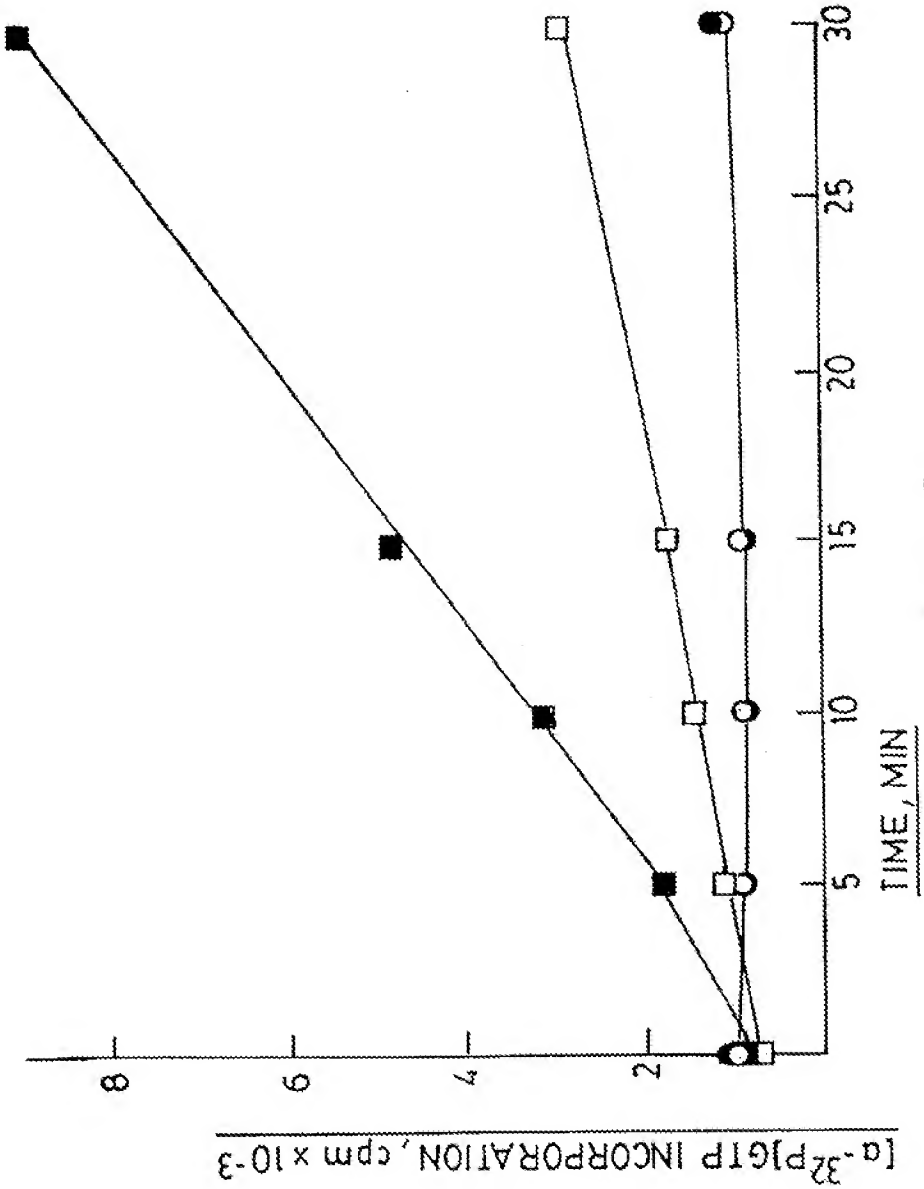

FIG.2

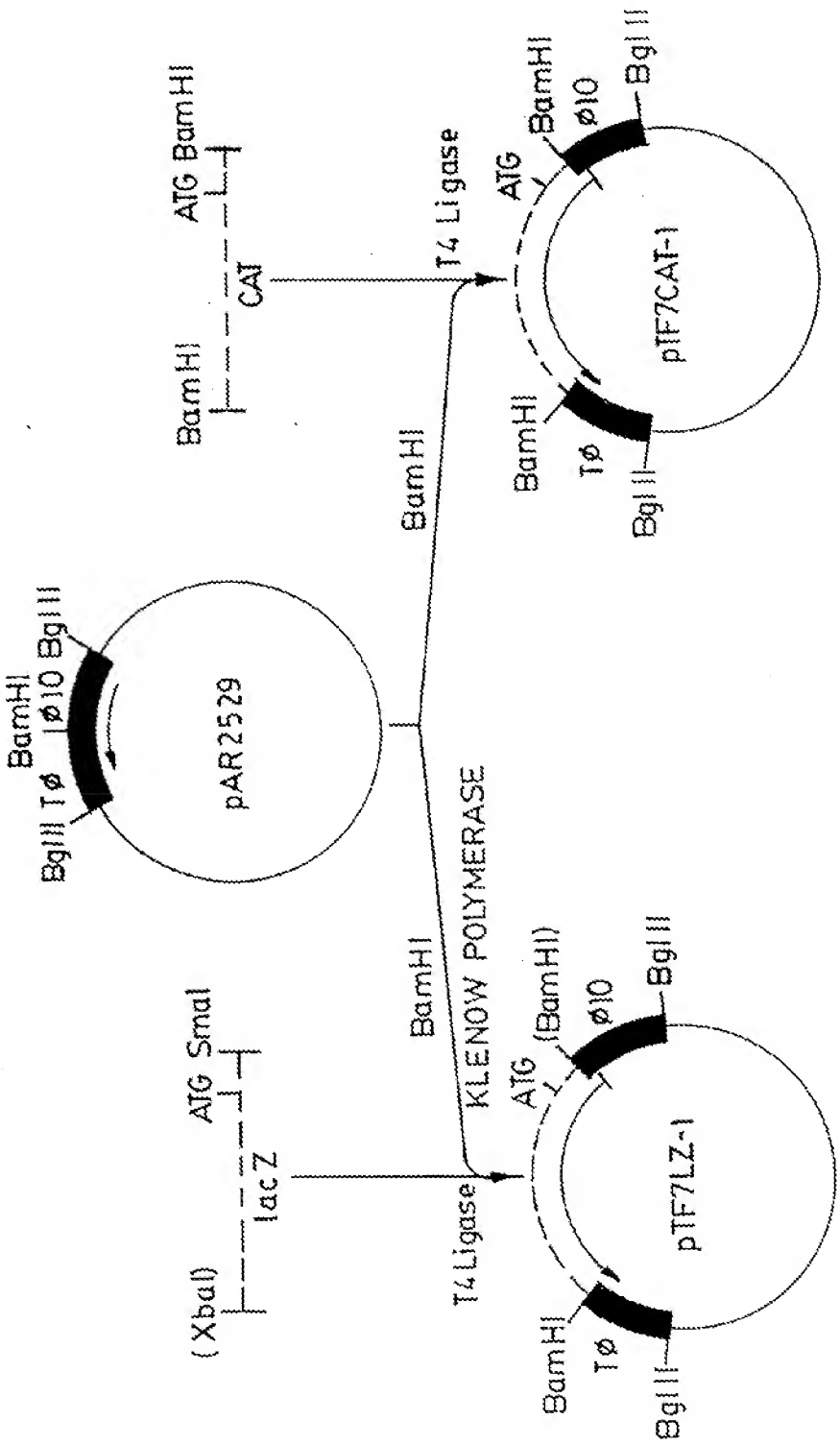
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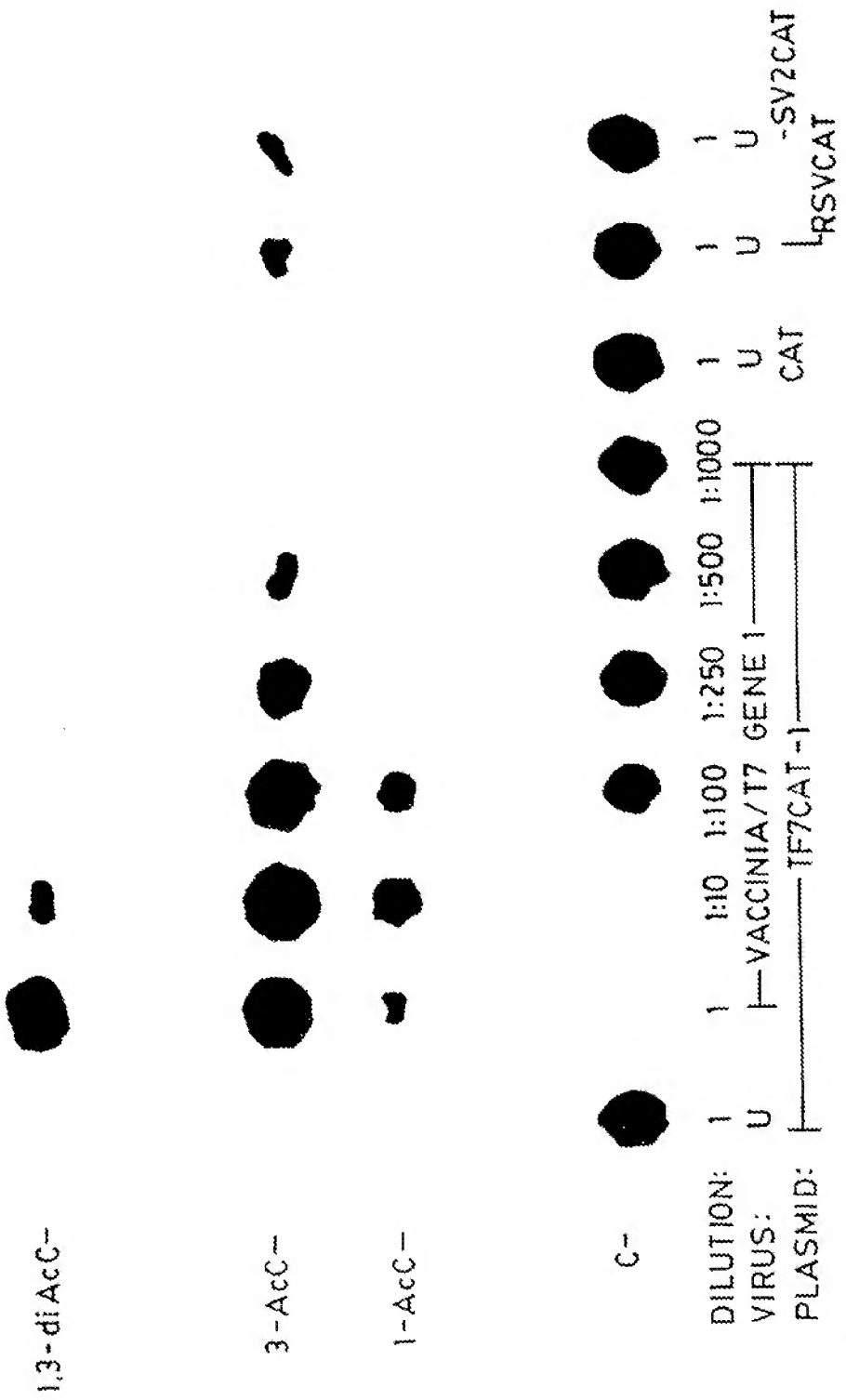
FIG. 3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3-AcC-
1-AcC-
C-
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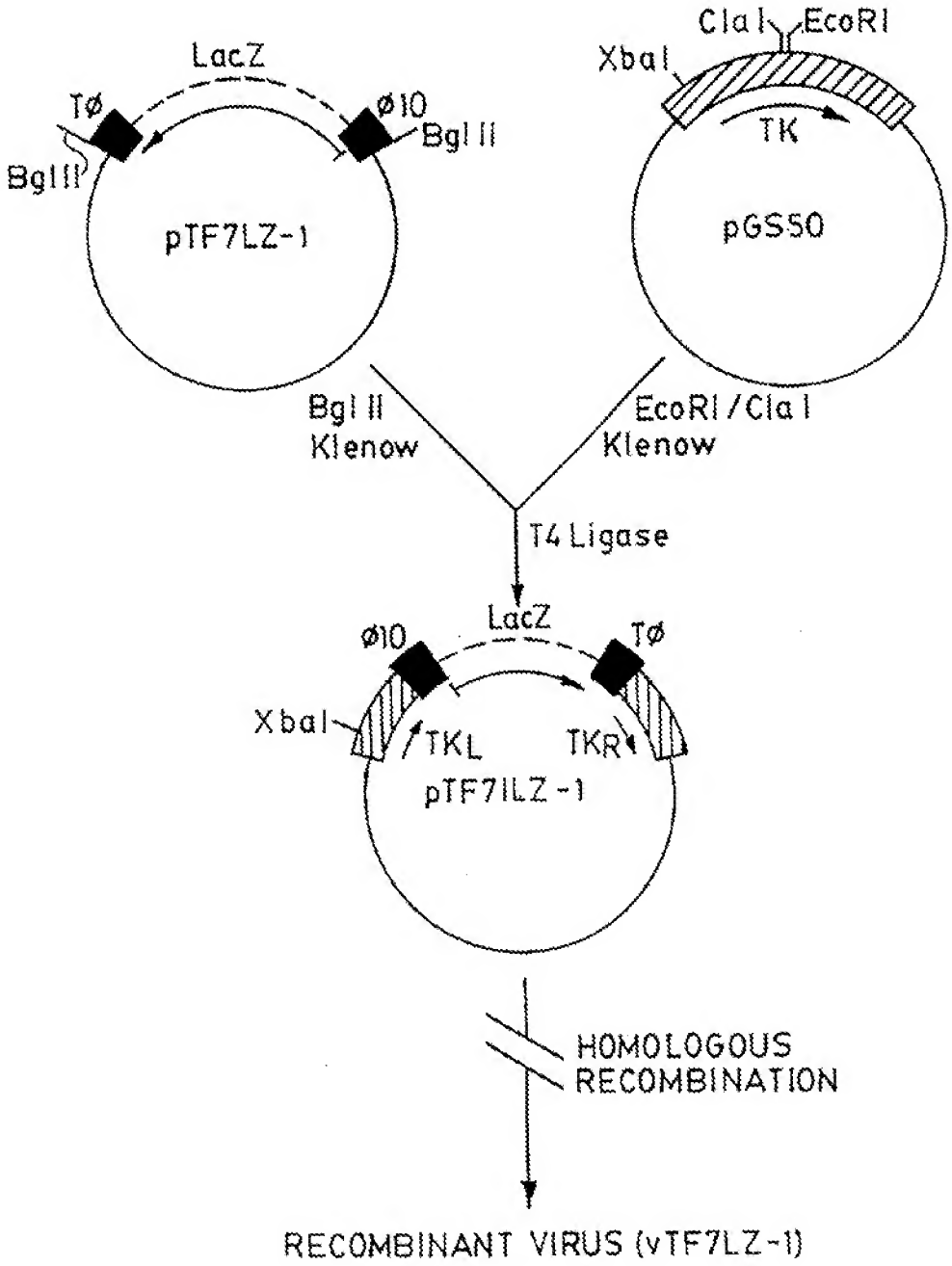
PLASMID: TF7CAT-1 CAT RSVCAT -SV2CAT

FIG.4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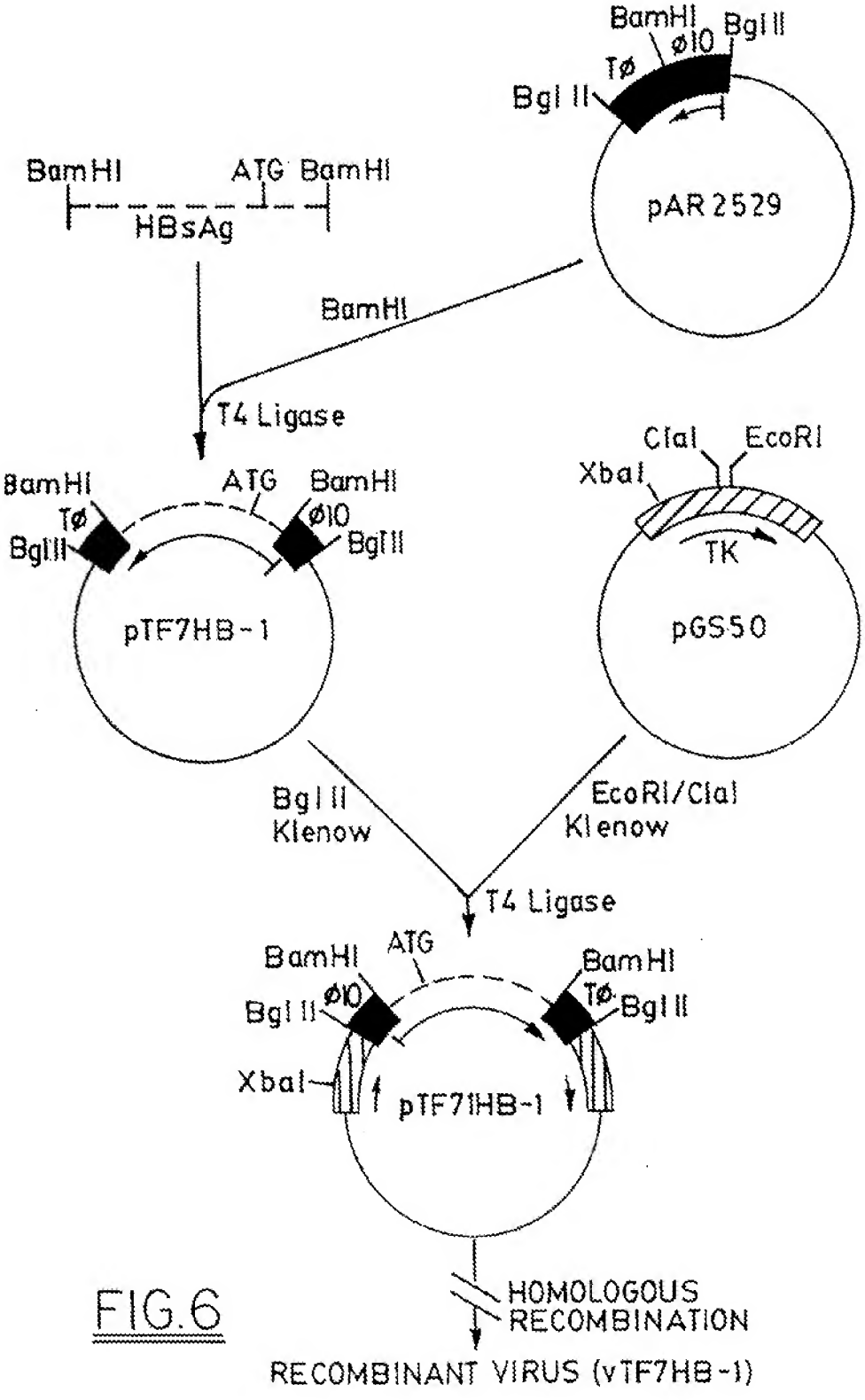
RECOMBINANT VIRUS (vTF7LZ-1)

FIG. 5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ATG
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BglII
ø10
Tø
BglII
XbaI
pTF71HB-1
HOMOLOGOUS
RECOMBINATION
RECOMBINANT VIRUS (vTF7HB-1)

FIG.6

PROKARYOTIC EXPRESSION IN EUKARYOTIC CELLS

This invention was made wholly or in part with funds provided by the U.S. Government. The U.S. Government has certain rights in this invention.

This application is a "continuation" of application Ser. No. 07/648,971, filed Jan. 31, 1991, now abandoned, which is a continuation-in-part of Ser. No. 07/582,489, filed Sep. 14, 1990, now abandoned, which is a continuation-in-part of Ser. No. 905,253 filed Sep. 8, 1986, now abandoned.

FIELD OF THE INVENTION

This invention relates generally to the expression of genes in a eukaryptic environment and more particularly, to a transient expression system that utilizes bacteriophage RNA polymerase in the presence of a DNA-based cytoplasmic virus to facilitate expression of the gene in the cytoplasm of the eukaroytic cell.

BACKGROUND OF THE INVENTION

Since the inception of microbiology and genetic engineering, there has been a desire to be able to transfer traits from one organism to another.

Recombinant DNA technology has made it possible to develop molecular cloning vectors that allow expression of heterologous genes in prokaryotic cells (cells of lower life forms without a nucleus) and eukaryotic cells (cells of higher organisms). Bacterial systems provide important advantages such as ease of use and high expression but impose a number of limitations for synthesis of eukaryotic proteins. In particular, correct folding, proteolytic processing, glycosylation, secretion, and subunit assembly may not occur or may occur incorrectly in bacteria. For these and other reasons, eukaryotic cells are preferred for expression of eukaryotic genes.

It has also been difficult to obtain expression of certain genes, especially those of lower organisms in cells of higher organisms. This has been true for many reasons including the fact that gene control mechanisms are often significantly different.

It has been known that T7 and certain other bacteriophage RNA polymerases, for example SP6, GH1 and T3, are single subunit enzymes with high catalytic activity and strict promoter specificity, which have found wide application for in vitro synthesis of RNA and as the basis for high-level gene expression systems in *Escherichia coli*. One potential problem with use of a prokaryotic RNA polymerase in a eukaryotic cell, however, is the requirement for mRNA to be processed, capped, methylated, and polyadenylated. Another potential problem concerns the observation that eukaryotic RNA polymerases are produced in the cytoplasm but are transported and subsequently localized in the nucleus. A system using T7 or other bacteriophage RNA polymerases would require localization of the enzyme in the cytoplasm to act on foreign genes contained within the vector also localized in the cytoplasm. Therefore, a transient system which could utilize the advantages of a bacteriophage RNA polymerase, which could function in an eukaryotic environment to facilitate the expression of a foreign gene, and which is simple, widely applicable, and highly efficient, is desirable.

SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that foreign genes encoding a single subunit RNA polymerase can be used in eukaryotic cells when the procedures and vectors of the present invention are employed.

In particular, the invention comprises a method of expressing a foreign gene in the cytoplasm of a eukaryotic environment comprising living eukaryotic cells. The method comprises incorporating a DNA-based cytoplasmic virus into the evironment along with a suitable carrier i.e. vector, encoding a single subunit RNA polymerase which is foreign to the carrier and to the cells, along with a suitable carrier comprising a functional cistron including a promoter responsive to the bacteriophage RNA polymerase. The cistron further includes the gene to be expressed and may include a phage RNA polymerase specific transcription termination sequence.

The vector and DNA-based cytoplasmic virus may be the same, and it is within the scope of the present invention to include novel DNA-based cytoplasmic viral vectors. A combined vector may include a DNA-based cytoplasmic virus containing a foreign gene encoding for a functional specific single subunit RNA polymerase and a DNA-based cytoplasmic virus comprising a functional cistron including a foreign gene sequence flanked by a bacteriophage promotor and perhaps a termination sequence which functions with the bacteriophage RNA polymerase.

More particularly, one embodiment of the present invention may comprise a vaccinia/T7 transient expression system. This illustrative system is designed for expression of a foreign gene. The foreign gene is inserted into a vector between bacteriophage RNA polymerase T7 promoter and terminator sequences. Expression of the gene is facilitated by another vector encoding a bacteriophage RNA polymerase i.e., T7, polymerase which very specifically initiates transcription of DNA to m-RNA and eventual production of foreign protein. The foreign genes expressed utilizing this illustrative system includes the HBsAg gene of hepatitis B Virus (HBV) and the prokaryotic genes lacZ and CAT.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing insertion of bacteriophage T7 gene 1 which encodes RNA polymerase into the genome of vaccinia virus. A 2.65 Kb BamHI fragment containing T7 gene 1 was excised from pAR1173 and inserted into the unique BamHI site of pGS53 to form pTF7-3. In the latter plasmid, the coding sequence for T7 RNA polymerase is downstream of the vaccinia P7.5 promoter and the chimeric gene is flanked by the left (TKL) and right (TKR) vaccinia TK sequences. DNA segments are not drawn to scale. CV-1 cells were infected with vaccinia virus and transfected with pTF7-3. After 48 hrs, the cells were harvested and the virus was plaqued on TK- cells in the presence of BUdR. Virus plaques were amplified and screened by dot blot hybridization to T7 gene 1 DNA.

FIG. 2 is a graph demonstrating the synthesis of T7 RNA polymerase. Extracts were prepared from uninfected cells O, cells infected with vaccinia virus ●, infected with vaccinia virus and transfected with pTF7-3 □, or infected with vTF7-3 ■ and assayed for T7 RNA polymerase. Incorporation of [α-^{32}P]) GTP into RNA that bound to DEAE-cellulose filters was measured.

FIG. 3 is a schematic diagram showing construction of plasmids containing target genes flanked by T7 promoter and terminator sequences. A 3.2 Kb DNA segment containing the lacZ gene with translation and termination codons was obtained by cleavage of pWS61 (provided by A. Majmdar, NIH) with XbaI, filling in the staggered end with the

Klenow fragment of DNA polymerase and deoxynucleoside triphosphates, and cleaving with SmaI. The fragment was then blunt-end ligated to pAR2529 which had been cleaved with BamHI and treated with Klenow fragment of DNA polymerase. The resulting plasmid, pTF7LZ-1 has the coding sequence for β-gal flanked by the T7 Ø10 promoter and TØ terminator. Similarly, a 0.7 Kb BamHI fragment from pGS30 containing the CAT gene was ligated to BamHI cleaved pAR2529 to form pTF7CAT-1.

FIG. 4 shows a comparison of transient expression systems. Cell lysates were prepared at 48 hr after infection with vTF7-3 (vaccinia/T7 gene 1) and/or transfection with the indicated plasmid and assayed for CAT. Samples were spotted on a silica gel plate and chromotographed. An autoradiograph is shown with the positions of chloramphenicol (C) and acetylated forms of chloramphenicol (AcC) indicated.

FIG. 5 shows the construction of plasmid pTFILZ-1 and recombinant vaccinia virus vTF7LZ-1 containing the chimeric target gene for *E. coli* β-galactosidase (lacZ).

FIG. 6 shows recombinant vacciniavirus vTF7HB-1 containing the hepatitis B virus HBsAg gene.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, a transient expression system has been discovered which for the first time utilizes highly efficient single subunit bacteriophage RNA polymerases, such as those from T7, SP6, GH1, and T3 viruses, in a eukaryotic environment. As previously discussed, the use of such RNA polymerases in eukaryotic environments has not been practical or possible due to supplemental requirements associated with expression of genes by such RNA polymerases. In accordance with the present invention, it has been discovered that the presence of a DNA based cytoplasmic virus will permit such a gene to be expressed in a eukaryotic environment.

The term "DNA-based cytoplasmic viruses", as used herein, are viruses that contain genetic material made up of DNA (versus an RNA virus) that when infecting a cell carries its genetic material into the cytoplasm of the target cell.

The DNA-based cytoplasmic viruses lack proper signals or apparatus to enter the nucleus of the infected cell, but contain all necessary information for transcription and replication in the cytoplasm, and thus provide some of the necessary components that contribute to expression of a foreign gene.

The major family of viruses which transcribe and replicate their DNA in the cytoplasm are the poxviruses; although certain Iridoviruses, such as swine fever virus, often transcribe and replicate much of their DNA in the cytoplasm and therefore may be used in accordance with the present invention.

Examples of genera of poxviruses within the scope of the present invention include orthopoxvirus, parapixvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus molluscipoxvirus and yatapoxvirus. More particularly, examples of species of poxviruses within the scope of the present invention include rabbit pox virus, cow pox virus, sheep fibroma virus, ectomelia (mouse pox virus), and vaccinia virus. Vaccinia virus is especially suitable for use in accordance with the present invention and further detailed discussion will refer to this virus. It is, however, to be understood that such a discussion in general similarly applies to the other cytoplasmic DNA viruses.

Vaccinia virus, the prototypal member of the poxvirus family, has a large linear double-stranded DNA genome that encodes an entire transcription system including RNA polymerase, capping/methylating enzymes, and poly(A) polymerase. Additional advantages of vaccinia virus include its large capacity for foreign DNA, genome stability, and wide vertebrate host range. These characteristics have been utilized in the development of vaccinia virus as a eukaryotic expression vector.

The RNA polymerase genes which may be used in accordance with the present invention include any RNA polymerase which will function in the cytoplasm of a eukaryotic cell in the presence of a DNA-based cytoplasm virus. For purposes of example, particularly suitable are RNA polymerase genes from bacteriophage, bacterial viruses, and especially the T7, SP6, GH1, and T3 viruses. For purposes of illustrating a preferred embodiment of the present invention, and not limitation, the T7 RNA polymerase gene will be discussed in detail. The T7 RNA polymerase gene is isolated from the prokaryotic (viral) T7 bacteriophage. The T7 bacteriophage infects bacteria, but not eukaryotic cells. The T7 RNA polymerase is highly specific for promotor sequences contained within the bacteriophage genome. Accordingly, it is understood that the chances of finding a similar sequence in eukaryotic or other DNA are very nominal. For a further discussion on the specificity and individual promotors recognized by the bacteriophage RNA polymerases see Chamberlin et al, *The Enzymes*, vol. 15, pp. 82–108 (1982); and Dunn et al, *J. Mol. Biol.* 166, pp. 477–535 (1983). It is understood that the discussion with respect to the T7 RNA polymerase generally applies to other RNA polymerases, especially the bacteriophage RNA polymerases mentioned above.

The foreign gene which is to be expressed in the eukaryotic environment may be almost any gene sequence. The gene must, however, be included, within a cistron that will function with the bacteriophage RNA polymerase as previously described. "Cistron", as used herein, is a gene including a transcription promoter sequence which permits the gene to be expressed. Optionally, the "cistron may include a termination sequence. As used herein, a "cistron" and a "functional genetic sequence" may be considered the same. Such a cistron therefore includes a promoter, an intermediate or foreign sequence, and may optimally have a termination sequence. The intermediate or foreign sequence may contain one or more genetic sequences or a plurality of codes encoding polypeptides including proteins from the transcribed corresponding m-RNA. For purposes of describing such an intermediate sequence each such individual code will be referred to herein as a "gene." The promoter of the cistron used in accordance with the present invention functions with the RNA polymerase, as previously described. A further detailed discussion will be directed to such cistrons beginning with a T7 promoter. However, it is understood that such a discussion is for purposes of illustration only and, not limitation. For example, other promoters which will function with a T7 RNA polymerase or with other bacteriophage RNA polymerases are within the scope of the present invention.

The carrier used to deliver the gene for the RNA polymerase or the cistron into the eukaryotic environment includes any suitable vector, such as a plasmid or a virus. It is understood that the terms "vector" and "carrier", as used herein, are intended to be interchangeable terms. In accordance with the present invention, a DNA-based cytoplasmic

virus in the eukaryotic environment permits the RNA polymerase gene to be expressed. The same virus may also act as a vector in accordance with the present invention, i.e. it may also be used to introduce the RNA polymerase gene or the cistron into the environment. Optionally a plasmid may be used as the vector or vectors to introduce the RNA polymerase gene and/or the cistron into the eukaryotic environment. When using a plasmid as the carrier of the foreign gene, a DNA-based cytoplasmic virus is needed to provide information required for expression of the RNA polymerase gene. The cytoplasmic DNA virus may also provide other functions including capping and polyadenalation of the RNA synthesized by the T7 RNA polymerase.

A better understanding of the present invention and of its many advantages will be had by referring to the following examples. It is understood however, that the procedures and examples described hereinafter are for purposes of illustration only, and that any changes or modifications which suggest themselves to one of ordinary skill in the art are within the scope of the present invention.

EXAMPLE I

Materials and Methods

1.0 ENZYMES

For the procedures described, enzymes were supplied by the companies indicated and used in accordance with their instructions. Restriction endonucleases were from Bethesda Research Laboratories, New England BioLabs or Boehringer Mannheim Biochemicals. The Klenow fragment of DNA polymerase I and T4 DNA ligase were from New England BioLabs. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals.

2.0 VIRUS AND CELLS

Vaccinia virus (strain WR) was originally obtained from the American Type Culture Collection, replicated in HeLa cells, and purified per the procedure of Macken et al., *DNA Cloning*, Vol. 2 , pp. 191–211, 1985. HeLa cells were grown in Eagle's medium supplemented with 5% horse serum. Human TK- 143 cells (Rhine et al., Int. J. Cancer, 1975, 15, pp 23–29) were grown in Eagle's medium with 10% fetal bovine serum (FBS) and 25 μg of 5-bromodeoxyuridine (BUdR) per ml. CV-1 monkey kidney cells were grown in Dulbecco's modified medium containing 10% FBS.

3.0 PLASMIDS

Plasmid pGS53 contains the vaccinia virus P7.5 promoter, unique BamHI and SmaI restriction sites for insertion of foreign genes, and thymidine kinase (TK) flanking sequences. It differs from the pGS20 vector, (Mackett et al., J. Virol, 1984, 49 pp 857–864), principally in the use of pUC13 plasmid (Messing, Methods Enzymol., 1983, 101C, pp 20–78) instead of pBR328 and TK flanking sequences derived from the Wyeth strain of vaccinia virus instead of the WR strain.

4.0 Preparation and Cloning of DNA

Recombinant plasmids were constructed and used to transform bacteria following the methods of Maniatis et al., Molecular Cloning, 1982. plasmids were prepared by the alkaline NaDodSO4 method as described by Birnboim and Doly, Nucleic Acids Res., 7, pp 1513–1523, and purified by CsCl/ethidium bromide equilibrium density gradient centrifugation. Plasmids were routinely checked by agarose gel electrophoresis to ensure that the majority of DNA was in the supercoiled configuration. DNA fragments were isolated from low-melting point agarose gels following the Elutip d (Schleicher and Schuell) method. DNA was extracted from purified virus as described in Mackett et al., 1. supra.

5.0 Isolation of Recombinant Virus

To isolate recombinant virus, CV-1 cells were infected with 0.05 plaque forming units (PFU) per cell of wild-type vaccinia virus and transfected with calcium phosphate precipitated plasmid as described previously, Mackett et al., 1. supra. TK^- recombinant virus plaques were isolated on TK^- 143 cell monolayers in the presence of BUdR (25 μg/ml). Recombinant virus plaques were distinguished from spontaneous TK- mutant virus by DNA:DNA dot blot hybridization. After two consecutive plaque purifications, recombinant virus was amplified by infecting TK^- 143 cell monolayers in the presence of BUdR and then large stocks were made in HeLa cells without selection.

6.0 Transient Assay Conditions

For standard assays (Cochran et al., Proc. Nat. Acad. Sci., 1985, 82 pp 19–23) CV-1 cells were grown to 80% confluency in 25 cm2 flasks (approximately 2.5×10^6 cells) and infected with either purified wild-type of recombinant vaccinia virus at a multiplicity of 30 PFU per cell. The virus was allowed to absorb for 30 minutes at 37° C. with occasional rocking of the plate. The inoculum was then removed and 1 ml of calcium phosphate-precipitated DNA (10 μg of recombinant plasmid and 10 μg of salmon sperm DNA) was added. After 30 minutes at room temperature, fresh medium containing 2.5% FBS was added and the flask was incubated at 37° C. Cells were harvested at 24 hours after infection and suspended in the indicated buffer.

When specified, care was taken to follow the transient expression conditions described by Gorman and co-workers. *Mol. Cell. Biol.* 1982, 2 pp 1044–1051. On the day prior to transfection, low passage number (less than 10 passages) CV-1 cells were plated at a density of 2.5×10^6 cells per 25 cm^2 flask and were refed with Fresh medium containing 10% FBS at 3 hours before transfection. A 2 minute glycerol shock was performed at 3.5 hours after transfection and cell lysates were prepared at 48 hours.

7.0 T7 RNA Polymerase Assay

Approximately 2.5×10^6 transfected or infected CV-1 cells were resuspended in 0.25 ml of 0.01M Tris-HCl (pH 7.6)/0.01M NaCl/1.5 mM $mgCl_2$ and Dounce homogenized. After centrifugation, 1.5 μl of cytoplasmic supernatant was assayed for T7 RNA polymerase at 37° C. in 0.025 ml mixtures containing: 40 mM Tris-HCl (pH 8.0)/8 mM $MgCl_2$/2 mM spermidine/30 mM NaCl/1 mM each of ATP, CTP, and UTP/5 uM [α-^{32}p]GTP/30 mM dithiothreitol/1 μg of pTFLZ-1 template/40 units of RNasin (Promega Biotech). At various times the reactions were stopped by addition of 0.05 ml of 50 mM EDTA/0.1% SDS/100 μg of proteinase K per ml and incubated for 60 minutes at 37° C. Samples were applied to DE 81 paper (Whatman) and washed three times for 5 minutes in 5% Na_2HP0_4, once with water and once with 95% ethanol. The samples were dried and counted in a scintillation spectrophotometer.

8.0 β-galactosidase (β-gal) Assay

Approximately 2.5×10^6 infected or transfected CV-1 cells were suspended in 1 ml of phosphate buffered saline, frozen and thawed three times and dispersed by sonication. The cellular debris was removed by centrifugation and the supernatant was assayed for β-gal activity using o-nitrophenyl-β-D-galactopyranoside as described by Miller, *Experiments in Molecular Genetics*, 1972, pp 352–355. After 30 minutes at 37° C., the reaction was stopped by addition of 1M Ca_2C0_3 and the yellow color was quantitated at an absorbance of 420 nm. β-gal activity was recorded as nmol of O-nitrophenol produced per 2.5×10^6 cells.

9.0 Chloramphenicol Acetyltransferase (CAT) Assay

Approximately 2.5×10^6 infected or transfected CV-1 cells were suspended in 0.2 ml of 0.25M Tris-HCl (pH 7.5). After freezing and thawing three times, the lysates were dispersed by sonication, and the suspension was assayed for enzyme activity as described by Mackett et al. 2., supra.

EXAMPLE II

Construction of Plasmids pGS53 and PGS50

Plasmid pGS53 is basically similar to pGS20 (Mackett, Smith and Moss, *J. Virol.* 49, 857–864) which also could have been used to construct a recombinant vaccinia virus that expresses T7 RNA polymerase gene 1. Unless otherwise indicated, all recombinant DNA steps were carried out by standard procedures such as those previously used for construction of pGS20 and those described by T. Maniatis, E. F. Fritsch and J. Sambrook *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1982. Plasmid pUC13 was cleaved with restriction endonuclease EcoRI, extracted with phenol-chloroform and ethanol precipitated. The linearized plasmid was then digested with nuclease S1 to remove the 5' overhanging nucleotides at the EcoRI site and again phenol-chloroform extracted and ethanol precipitated. To ensure that the DNA ends were blunt, the plasmid was then incubated with all four deoxyribonucleoside triphosphates and the Klenow fragment of DNA polymerase. The DNA was purified once more by phenol-chloroform extraction and ethanol precipitated and ligated to a 1,800 base pair DNA fragment containing the vaccinia virus thymidine kinase gene. The latter 1,800 base pair DNA fragment was produced by digesting the HindIII J fragment of vaccinia virus (New York City Board of Health strain from Wyeth Laboratories) with PvuII and was purified by agarose gel electrophoresis. Transformation competent *E. coli* were transformed with the ligated DNA and transformants were selected and grown and the plasmid designated pGS50 was amplified and purified. pGS50 that had been cleaved with EcoRI and phosphatase treated was ligated to an agarose gel purified fragment of approximately 290 base pairs, that contains the vaccinia virus P7.5 promoter with downstream BamHI and SmaI sites, which was obtained by digesting pGS19 (Mackett, Smith and Moss, *J. Virol.* 29, 857–864) with EcoRI. Transformation competent *E. coli* were transformed with the ligated DNA and transformants were selected and grown and the plasmid designated pGS53 was amplified and purified.

EXAMPLE III

Construction of Recombinant Plasmid pAR2529

Plasmid pAR2529 contains the bacteriophage T7 promoter, Ø10, and terminator, TØ, and was constructed as follows. Synthetic BamHI linkers, CGGGATCCCG, were ligated to a fragment of T7 DNA (nucleotides 22,880–22,928) containing the Ø10 promoter for T7 RNA polymerase digested with BamHI and inserted into the BamHI site of pBR322. The fragment extends from nucleotides −23 to +26 relative to the start of the RNA and is oriented so that transcription from the Ø10 promoter is directed counterclockwise, opposite to transcription from the tetracycline promoter. The upstream BamHI site was converted to a BglII site by partial digestion with BamHI, removal of the 5' overhang by filling in with the Klenow fragment of *E. coli* DNA polymerase in the presence of all four deoxyribonucleotides, adding the linker GAGATCTC, cleaving with BglII, and re-ligating. A fragment of T7 DNA containing TØ (nucleotides 24,306–24,228, where transcription terminates at nucleotide 24,209) was joined to the downstream BamHI cloning site through the sequence GGATCCGG'-TØ-CCG-GATCGAGATCTCGATCC, where the final C is nucleotide 375 in the BamHI site of pBR322. The downstream linker contains a BglII site, so the entire Ø10-BamHI-TØ fragment can be removed from this plasmid as a BglII fragment for transfer to other vectors.

EXAMPLE IV

Construction of Plasmid DTF7-3

Plasmid pTF7-3 (A.T.C.C. No. 67202) was constructed by inserting a 2.65 Kb DNA fragment, containing the entire T7 gene 1 coding region for T7 RNA polymerase, into the BamHI site of pGS53.10 µg of plasmid pAR1173 (Davanloo, P., Rosenberg, A. H., Dunn, J. J., and Studier, F. W. (1984) *PNAS USA* 81, 2035–2039.) was digested with BamHI, separated by gel electrophoresis, and the 2.65 Kb T7 RNA polymerase DNA fragment isolated following the Elutip-d (Schleicher and Schuell) method. 5 µg of pGS53 DNA was cleaved with BamHI and gel purified as above. The 2.65 Kb T7 RNA polymerase DNA fragment was ligated to pGS53 and used to transform competent *E. coli* cells following procedures as outlined in Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1982.

EXAMPLE V

Construction of Plasmid pTF7LZ-1

Plasmid pTF7LZ-1 was constructed by inserting an *E. coli* lacZ DNA fragment containing a translation initiation codon, ATG, inframe with the lacZ coding sequence into the BamHI site of pAR2529. The lacZ DNA fragment was isolated from the plasmid pWS61 (obtained from Alokes Maoumdar, NIH, unpublished) as a 3.2 Kb XbaI/Aha3 fragment which contains the following DNA sequence upstream from the 9th codon of the lacZ structural gene: TCTAGATTATTTGCATACATTCAAT-CAATTGTTATCTAAGGAAATACTT ACATATGGT-TCGTGCTAACAAACGCAACGAGGCT CTAC-GAATCGCGATAAGCTAGCTTGGGGGGATCCC.

The 5' overhangs of the 3.2 Kb XbaI/Aha3 fragment were filled in with the Klenow fragment of *E. coli* DNA polymerase in the presence of all four deoxyribonucleotides. Plasmid vector pUC18 was cleaved with BamHI, the 5' overhangs filled in as before, and the blunt-ended lacZ fragment inserted by ligation following standard cloning techniques (Maniatis et al., 1982). The resulting plasmid was named pGa12. 10 µg of pGa12 DNA was digested with XbaI, 5' overhang filled in with the Klenow fragment, cleaved with SmaI, and separated by agarose gel electrophoresis. The 3.2 Kb lacZ fragment was isolated by the Elutip-d method. 10 µg of plasmid pAR2529 DNA was digested with BamHI, made blunt with the Klenow fragment, 5' terminal phosphates removed by treatment with calf intestine alkaline phosphatase, phenol/chloroform extracted once blunt end-ligated to the lacZ fragment, and aliquots of the ligation mixture were used to transform competent *E. coli* HB101 cells following standard techniques. Plasmid clones with lacZ coding sequence in the positive (productive) orientation with respect to the T7 promoter, Ø10, were designated pTF7LZ-1.

EXAMPLE VI

Construction of Plasmid pTF7CAT-1

Plasmid pTF7CAT-1 was constructed by inserting a DNA fragment containing the CAT-coding sequence derived from pBR328 into the BamHI site of pAR2529. The CAT gene was isolated as a 770-bp TaqI DNA fragment containing the CAT coding sequence separated from its endogenous start site from pSR328 and cloned into the AccI site of pUC7. Since pUC7 contains BamHI sites closely flanking the AccI sites, the CAT gene was excised with BamHI and inserted into pGS20. (Mackett, M., Smith, G. L., and Moss, B. PNAS USA 49:857–864.). The resulting plasmid was designated pGS30. 10 μg of pGS30 DNA was digested with BamHI and separated by agarose gel electrophoresis. The 0.8 Kb CAT fragment was isolated using the Elutip-d method. 10 μg of pAR2529 DNA was digested with BamHI, 5' terminal phosphates removed by using calf intestine alkaline phosphatase, phenol/chloroform extracted once, ligated to the CAT gene fragment, and aliquots of the ligation mixture used to transform competent *E. coli* HB101 cells following standard techniques. Plasmid clones with the CAT coding sequence in the positive orientation with respect to the T7 promoter, Ø10, were designated pTF7CAT-1.

EXAMPLE VII

Construction of Plasmid DTF7HB-1

Plasmid pTF7HB-1 was constructed by inserting a DNA fragment containing the coding sequence for hepatitis B surface antigen (HBsAg) gene into the BamHI site of pAR2529. HBsAg coding gene sequence was isolated as a 0.9 Kb BamHI DNA fragment from pHBs4 (Smith, G. L., Mackett, M., and Moss, B. Nature 302: 490–495, 1983). 10 μg of phBs4 DNA was digested with BamHI and the DNA fragments separated by agarose gel electrophoresis. The 0.9 Kb HBsAg fragment was isolated using the Elutip-d method. 10 μg of pAR2529 DNA was digested with BamHI, 5' terminal phosphates removed by calf intestine alkaline phosphatase treatment, phenol/chloroform extracted once, ligated to the HBsAg gene fragment, and aliquots of the ligation mixture used to transform competent *E. coli* cells following standard techniques. Plasmid clones with the HBsAg coding sequence in the positive orientation with respect to the T7 promoter, Ø10, were designated pTF7HB-1.

EXAMPLE VIII

Construction of Plasmid DTF7LZ-1

Plasmid pTF7LZ-1 was constructed by inserting a 3.4 Kb DNA fragment, containing the T7-promoter-lacZ gene T7 terminator BglII fragment from pTF7LZ-1, into the ClaI/EcoRI site of pGS50. 10 μg of pTF7LZ-1 was digested with BglII, 5' protruding ends filled in with the Klenow fragment of *E. coli* DNA polymerase in the presence of all four deoxyribonucleotides, DNA fragments separated by agarose gel electrophoresis, and the 3.4 Kb fragment isolated by the Elutip-d method. 10 μg of plasmid pGS50 was digested with EcoRI, 5' protruding ends filled in with the Klenow fragment, cleaved with ClaI, treated with calf intestine alkaline phosphatase, and phenol/chloroform extracted. The 3.4 Kb T7 promoter-lacZ gene-T7 terminator fragment was ligated to this preparation of pGS50 and aliquots of the ligation mixture used to transform competent *E. coli* cells following standard techniques. Plasmid clones with the lacZ coding sequence in the same orientation as the thymidine kinase (TK) coding sequence were designated pTF7LZ-1.

EXAMPLE IX

Construction of Plasmid pTF7HB-1

Plasmid pTF7HB-1 was constructed by inserting a 1.1 Kb DNA fragment, containing the T7 promoter-HBsAg gene-T7 terminator BglII fragment from pTF7HB-1, into the ClaI/EcoRI site of pGS50. 10 μg of pTF7HB-1 was digested with BglII, the 5' protruding ends filled in using the Klenow fragment, DNA fragments separated by agarose gel electrophoresis, and the 1.1 Kb fragment purified using the Elutip-d method. 10 μg of pGS50 DNA was cleaved with EcoRI, 5' protruding ends filled in with the Klenow fragment, digested with ClaI, treated with calf intestine alkaline phosphatase, and phenol/chloroform extracted. The 1.1 Kb DNA fragment was ligated to the preparation of pGS50 and aliquots of the ligation mixture were used to transform competent *E. coli* cells. Plasmid clones with the coding sequence of HBsAg in the same orientation as the TK coding sequence were designated pTF7HB- 1.

EXAMPLE X

Construction of Recombinant Virus vTF7-3

Construction of the recombinant virus, vTF7-3 (A.T.C.C. No. VR 2153) was done using the following procedures. CV-1 cells were infected with 0.05 plaque forming units (PFU) per cell of wild-type vaccinia virus and transfected with calcium phosphate-precipitated pTF7-3 DNA following procedures described previously (Mackett, M., Smith, G. L., and Moss, DNA Cloning, Vol. 2, ed Glover, D. M. IRL Press, Oxford, pp. 191–211 1985). Recombinant virus was formed by homologous recombination into the TK locus and TK^{-} virus was selected by plaque assay on TK^{-} 143 cell monolayers in the presence of BUdR (25 ug per ml). TK-recombinant virus plaques were distinguished from spontaneous TK^{-} mutant virus by DNA:DNA dot blot hybridization (ibid). After two consecutive plaque purifications the recombinant virus, vTF7-3, was amplified by infecting TK-143 cell monolayers in the presence of BUdR and then large stocks were made in HeLa cells without selection.

EXAMPLE XI

Construction of Recombinant Viruses vTF7LZ-1 and vTF7HB-1

Recombinant viruses vTF7LZ-1 (A.T.C.C. No. VR 2152) and vTF7HB-1 (A.T.C.C. No. VR 2154) were constructed following the same procedures as outlined above using plasmid vectors pTF7LZ-1 and pTF7HB-1, respectively.

EXAMPLE XII

Construction of Recombinant Vaccinia Virus Containing a Chimeric Bacteriophage T7 RNA Polymerase Gene

Procedures for the insertion and expression of foreign genes in vaccinia virus have been described in detail, Mackett et al., I and 2, supra. Vaccinia virus promoters are required to regulate transcription of the DNA which is introduced by homologous recombination into the 185,000 bp linear double-stranded DNA genome. To facilitate the use of vaccinia virus as a vector, a series of plasmids were made

that contain a vaccinia virus promoter, restriction endonuclease sites for insertion of foreign DNA, and flanking vaccinia TK (thymidine kinase) sequences to direct recombination into the TK locus of the genome. For this study we used the plasmid pGS53 which contains a promoter termed P7.5 with early and late regulatory signs as described by Cochran et al., *J. Virology* 1985, 53, pp 30–37) to permit continuous expression of foreign genes. A 2.65 kilobase pair (Kb) DNA fragment, containing the entire T7 gene 1 coding region for T7 RNA polymerase, was excised with BamHI from plasmid pARI173 described by Davanloo et al., *Proc. Nat. Acad.*, 1984, 81, pp 2035–2039, and inserted into the unique BamHI site of pGS53 as shown in FIG. 1. A plasmid designated pTF7-3, with the vaccinia promoter and T7 RNA polymerase in proper orientation, was isolated from transformed *E. coli*. Plasmid pTF7-3 was used to transfect cells that were infected with vaccinia virus and then TK^{-} recombinant virus plaques were selected. Correct insertion of the T7 RNA polymerase gene into the genome of vTF7-3 was confirmed by DNA blot hybridization.

EXAMPLE XIII

Expression of T7 RNA Polymerase in Mammalian Cells

Previous studies, Cochran et al., 1., supra, indicated that plasmids containing genes under control of a vaccinia virus promoter are specifically transcribed in cells infected with vaccinia virus. To determine whether active T7 RNA polymerase would be synthesized when vaccinia virus infected cells were transfected with the plasmid pTF7-3, T7 RIgA polymerase activity in cell lysates was assayed using a DNA template containing a T7 promoter. Initial experiments established that RNA polymerase activity measured with this template was not increased after vaccinia virus infection alone, as shown in FIG. 2. When vaccinia virus infected cells were also transfected with pTF7-3, however, a significant increase in activity was observed (FIG. 2). Additional experiments demonstrated that T7 RNA polymerase activity was not detected when uninfected cells were transfected with pF7-3 or when infected cells were transfected with a plasmid containing the T7 gene 1 without a vaccinia promoter.

To demonstrate whether higher levels of T7 RNA polymerase would be expressed when the T7 gene 1 under control of a vaccinia promoter was integrated into the vaccinia virus genome the following procedures were followed. As shown in FIG. 2, vTF7-3 infected cell extracts contained several times more T7 RNA polymerase activity than was present in cells that had been transfected with pTF7-3 in the presence of wild-type vaccinia virus. This quantitative difference between recombinant virus and transient expression systems was consistent with previous observations.

EXAMPLE XIV

Construction of Plasmids Containing Target Genes with T7 Promoters

To determine whether bacteriophage T7 RNA polymerase made under control of vaccinia virus can function in mammalian cells, we constructed plasmids containing target genes flanked by T7 promoter and termination regulatory elements. Plasmid pAR2529 (A. H. Rosenberg, J. J. Dunn and F. W. Studier) contains the ϕ10 promoter separated by a unique BamHI site from the T7 terminator T$_{\phi}$, which has a potential stem-loop structure followed by a run of thymidylate residues. As targets, we chose the *E. coli* β-gal gene (called lacZ) and the CAT gene derived from the TN9 transposon. These genes are ideal for expression systems because simple and quantitative assays are available for the enzyme products and there is no detectable background activity in mammalian cells. The lacZ or CAT gene, each with an associated ATG translation initiation codon, was inserted into the unique BamHI site of pAR2529, as shown in FIG. 3. Plasmids with lacZ and CAT in the correct orientation were designated pTF7LZ-1 and pTF7CAT-1, respectively.

EXAMPLE XV

Transient Expression of β-gal

Previously, cells infected with vaccinia virus and transfected with a plasmid containing the T7 gene 1 under control of a vaccinia virus promoter synthesized T7 RNA polymerase were discussed. It was further determined that vaccinia virus infected cells would express β-gal if they were transfected with plasmids containing the T7 gene 1 under control of a vaccinia promoter and the lacZ gene under control of a T7 promoter. Transient expression depends on vaccinia virus regulated synthesis of T7 RNA polymerase, the intracellular functioning of the T7 RNA polymerase, the production of translatable m-RNA from a T7 promoter, and the synthesis of a prokaryotic enzyme. As shown in Table 1, β-gal was detected in cell lysates. Omission of either vaccinia virus or the plasmid containing the T7 RNA polymerase gene prevented expression of β-gal. Negative results also were obtained when, either the T7 gene 1 or lacZ gene was oriented oppositely with respect to the vaccinia or T7 promoter, respectively.

In both experiments above, the T7 gene 1 and the lacZ gene were transcribed from plasmids. Since more T7 RNA polymerase is made when gene 1 is integrated into vaccinia virus, as shown in FIG. 2, higher amounts of β-gal are produced if cells are infected with recombinant vaccinia virus vTF7-3 and then transfected with the lacZ plasmid pTFLZ-1. As shown in Table 1, more than twice as much β-gal was made when T7 RNA polymerase was expressed by a recombinant virus than from a plasmid.

The expression of β-gal under control of T7 and vaccinia virus promoters was further determined. The vaccinia virus promoter used, P7.5, was the same as that regulating expression of T7 gene 1. When cells were infected with vaccinia virus and transfected with the plasmid containing the β-gal gene under control of the vaccinia promoter, β-gal activity was about 5% of that obtained with the vaccinia/T7 transient system. In fact, the level of β-gal obtained with the vaccinia/T7 transient system was higher than that obtained even when the β-gal gene with the P7.5 promoter was inserted into the vaccinia virus genome and the recombinant virus was used to infect cells as shown in Table 1. In these experiments, substantial amounts β-gal were made without changing the pyrimidine, at the −3 position relative to the translation initiation codon, to a purine so as to fit the eukaryotic consensus sequence shown by Kozak, *Nucleic Acids Res.*, 12, pp 3873–3893).

EXAMPLE XVI

Transient Expression of CAT

It is important to compare the vaccinia/T7 transient expression system of the present invention with a more conventional system which is used in mammalian cells.

Since CAT is the most common target gene used in mammalian cells for comparison of expression levels, experiments similar to those performed with β-gal were repeated and are shown in Table 1. As in the case of β-gal, it was observed that transient expression of CAT from the T7 promoter was higher when the T7 gene 1 was integrated into vaccinia virus than when it was co-transfected on a second plasmid. Also, expression was observed to be much higher in the vaccinia virus/T7 hybrid system of the present invention in comparison to when the CAT gene was expressed directly from the vaccinia promoter.

For further comparison, CV-1 cells were transfected with pSV2CAT or pRSVCAT which contain the same CAT DNA fragment derived from the TN9 transposon as pTF7CAT-1 and either the enhancer and promoter from the Rous sarcoma virus (RSV) long terminal repeat or the simian virus 40 (SV40) early region. In order not to prejudice the results in favor of the vaccinia/T7 system, previously described experimental conditions for expression of pSV2CAT and pRSVCAT were employed. Thus, low passage CV-1 cells and glycerol boosting were used and cell lysates were made at 48 hours after transfection. The extracts were diluted and tested for CAT activity. As shown in the autoradiogram in FIG. 4, several hundred times more CAT was made in the vaccinia/T7 system than with either pSV2CAT or pRSVCAT. More quantitative results obtained by scintillation counting indicated that 4560 nmol of chloramphenicol were acetylated per 2.5×10^6 cells using the vaccinia/T7 system compared to only 6.8 with pSV2CAT or 9.1 with pRSVCAT. This difference was even greater when glycerol boosting was omitted and cells were lysed at 24 hours after transfection.

TABLE 1

Transient Expression of β-gal and CAT

Virus	Plasmid 1		Plasmid 2		Expression	
Vaccinia	Promoter	Gene	Promoter	Gene	β-gal	CAT
WT	VV	T7 1	T7	lacz	3100	
	VV	T7 1	T7	lacz	0	
WT			T7	lacz	0	
T7 gene 1			T7	lacz	3406	
WT			VV	lacz	137	
lacZ					480	
WT	VV	T7 1	T7	CAT		1650
	VV	T7 1	T7	CAT		0
WT			T7	CAT		0
T7 gene 1			T7	CAT		4330
WT			VV	CAT		300
CAT						1430

CV-1 cells were uninfected or infected with wild-type (WT) or recombinant (T7 gene 1, lacZ, *or* CAT) vaccinia virus. The recombinant viruses had the foreign gene under control of the vaccinia virus P7.5 promoter and inserted into the TK locus. Uninfected or infected cells were transfected with 1 or 2 plasmids containing either the T7 gene 1, β-gal gene or CAT gene under control of the vaccinia virus P7.5 promoter (VV) or the T7 010 promoter. Cells were harvested after 24 hours and lysates were assayed for β-gal or CAT. Expression is given as nmol of product formed in 30 minutes per 2×10^6 cells.

Utility of the present system is further shown by demonstrating that higher levels of expression can be obtained by incorporating the chimetic target gene into vaccinia virus. Construction of recombinant viruses containing the chimetic target genes for *E. coli* β-galactosidase (lacZ), and hepatitis B surface antigen (HBsAG) is shown in FIGS. **5** and **6**, respectively. Target genes lacZ and HBsAg were flanked by T7 promoter and terminator sequences. These chimeric fragments were shuttled as cassettes and inserted into the TK DNA sequence of pGS50. The resulting recombinant viruses, vTF7LZ-1 and vTF7IHB-1, now contain target genes lacZ and HBsAg, respectively, under the control of T7 promoter.

Furthermore, co-infection of cultured mammalian cells by recombinant vaccina virus that produces T7 RNA polymerase (vTF7-3) and either vTF7LZ-1 or vTF7HB-1 results in specific high-level expression of β-gal or HBsAg. Comparison of vaccinia/T7 mixed infection expression levels to those previously obtained with recombinant vaccinia viruses is shown in Table 2. Expression with the vaccinia/T7 β-gal is approximately 5-fold higher than with a vaccinia recombinant containing lacZ under the control of the vaccinia virus P7.5 promoter inserted in the TK locus. The level of HBsAg production using the vaccinia/T7 system is also higher than the corresponding single vaccinia recombinant. CV-1 cells were infected or co-infected with recombinant (T7 gene 1, lacZ, or HBsAg) vaccinia virus(es) at a multiplicity of 10 PFU of virus per cell. The recombinant viruses had the foreign gene under control of either the vaccinia virus P7.5 promoter (VV) or the T7 promoter and inserted into the TK locus. Cells were harvested after 24 hours and cell lysates and media were assayed for β-gal or HBsAg. Expression of β-gal is given as total nmol of product formed in 30 minutes per 2.5×10^6 cells. Expression for HBsAg is given as total CPM$\times10^3$ obtained from a radio-radioimmunoassay immunoassay (AUSRUA II, Abbott) per 2.5×10^6 cells. Accordingly, the novel system of the present invention directs high-level expression of target genes.

TABLE 2

Mixed Infection Expression of β-gal and HBsAg

Virus 1		Virus 2		Expression	
Promoter	Gene	Promoter	Gene	β-gal	HBsAg
VV	T71			0	0
T7	lacz			0	
VV	T7 1	T7	lacz	62,000	
VV	lacz			12,000	
T7	HBs				0
VV	T71	T7	HBs		1225
VV	HBs				800

Expression of eukaryotic genes in prokaryotes and vice versa is known in the art. However, this generally involves only exchanges in coding sequences. The transfer of a transcription system from a prokaryote to a eukaryote, as disclosed in the present invention, opens up new opportunities for regulating gene expression. The present invention has accomplished integration of a gene which encodes a functional bacteriophage RNA polymerase into a virus with specificity for eukaryotic cells by utilizing a single subunit RNA polymerase characterized by stringent promoter specificity, and cytoplasmic DNA virus that encodes its own RNA modifying enzymes.

Transcription of the T7 RNA polymerase gene in vaccinia virus infected cells is accomplished by the vaccinia RNA polymerase and therefore is dependent on the fusion of the bacteriophage gene to a vaccinia promoter. Expression of T7 RNA polymerase could be obtained either by transfecting vacinia virus infected cells with a plasmid containing the chimeric gene or by integrating the gene into a nonessential site within the genome of the vaccinia virus. Recombinant viruses were stable, could be grown to high titer and

produced higher levels of T7 RNA polymerase than cells transfected with the plasmids.

The target genes chosen for expression by T7 RNA polymerase were cloned into the plasmid at a unique restriction site separating a T7 promoter from a T7 terminator. For these studies, the target genes (lacZ and CAT) had associated translational initiation codons, but other plasmid vectors that supply the ATG and appropriate flanking nucleotides could be used for production of fusion proteins. The key step was to transfect these plasmids into cells that were infected with the vaccinia virus recombinant which expressed the T7 RNA polymerase gene. The synthesis of β-gal and CAT was compared by the vaccinia/T7 expression system of the present invention to that which occurred with a straight vaccinia transient expression system (in which the target gene has a vaccinia promoter) and to a conventional transient expression system (in which either the enhancer and promoter from the long terminal repeat of Rous sarcoma virus or the early region of SV40 were used). The vaccinia/T7 system was 15 to 20 fold more efficient than the straight vaccinia system and 400 to 600 fold more efficient than the conventional system.

The greater efficiency of the vaccinia/T7 transient system of the present invention when compared to that of more conventional system may be attributed to several factors. First, since it is possible to infect tissue culture cells synchronously with vaccinia virus, all cells may have T7 RNA polymerase. Moreover, T7 RNA polymerase is a very active enzyme with a 5 fold faster elongation rate than that of *E. coli* RNA polymerase. It would appear that the bacteriophage enzyme is able to function within the eukaryotic environment. In addition, since the vaccinia virus RNA modifying enzymes and presumably T7 RNA polymerase are localized in the cytoplasm, the transfected plasmid does not have to enter the nucleus for transcription and the mRNA produced does not have to be processed and transported back to the cytoplasm for translation.

Current knowledge in the field of vaccinia virus expression vectors should be directly applicable to the expression system of the present invention. For example, there is abundant evidence that eukaryotic proteins made in vaccinia virus infected cells are properly processed, glycosylated, and transported to the plasma membrane. In addition, because of the wide host range of vaccinia virus, a variety of vertebrate cells of mammalian and avian origin are suitable. The T7 promoter is especially versatile because of its use for in-vitro synthesis of translatable mRNA and in prokaryotic expression vectors. Development of the vaccinia/T7 hybrid virus system makes it possible to use previous or slightly modified plasmid vectors for a third purpose: efficient expression of genes in eukaryotic cells. We have concentrated our initial efforts on the application of the vaccinia/T7 system for transient expression of target genes in plasmids because of its simplicity and potentially wide application in this configuration. However, even higher levels of expression may be possible when both the T7 RNA polymerase gene and the target gene are carried by vaccinia virus vectors.

It is to be understood that the invention is by no means limited to the specific examples which have been illustrated and described herein and that various modifications thereof that may suggest themselves to one of ordinary skill in the art are within the scope of the present invention as defined by the appended claims.

We claim:

1. A method of expressing a gene in a eukaryotic cell comprising incorporating into said cell:

a DNA-based cytoplasmic virus;

a first carrier including a first gene encoding a bacteriophage RNA polymerase selected from the group consisting of T7, SP6, GH1 and T3 RNA polymerase, said first gene being foreign to the carrier and to the cell, and a first promoter sequence which initiates expression of said first gene; and

a second carrier comprising a functional cistron comprising a second foreign gene including a second promoter sequence responsive to said bacteriophage RNA polymerase, such that upon recognition of said RNA polymerase, said second promoter initiates expression of the second foreign gene in the eukaryotic cell.

2. The method of claim **1**, wherein said virus is selected from the group consisting of poxvirus and iridovirus.

3. The method of claim **2**, wherein said virus is poxvirus.

4. The method of claim **3**, wherein said poxvirus is selected from the group consisting of orthopoxvirus, parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, molluscipoxvirus and yatapoxvirus.

5. The method of claim **4**, wherein said poxvirus is orthopoxvirus.

6. The method of claim **5**, wherein said orthopoxvirus is vaccinia.

7. The method of claim **1**, wherein said first carrier is a plasmid.

8. The method of claim **7**, wherein said first promoter is a poxvirus promoter.

9. The method of claim **8**, wherein said first gene is the T7 RNA polymerase gene.

10. The method of claim **9**, wherein said plasmid is pTF7-3 having A.T.C.C. Designation Number 67202.

11. The method of claim **1**, wherein said second carrier is a plasmid.

12. The method of claim **1**, wherein said second promoter is a T7 promoter.

13. The method of claim **11**, wherein said second foreign gene is a eukaryotic gene.

14. The method of claim **11**, wherein said cistron further includes a terminator sequence.

15. A method of expressing a gene in a eukaryotic cell comprising incorporating into said cell:

a DNA-based cytoplasmic virus including a first gene encoding a bacteriophage RNA polymerase selected from the group consisting of T7, SP6, GH1 and T3 RNA polymerase, which first gene is foreign to the virus and to the cell, and a first promoter sequence which initiates expression of the first gene; and

a carrier comprising a functional cistron comprising a second foreign gene including a second promoter sequence responsive to said bacteriophage RNA polymerase, such that upon recognition of said RNA polymerase, said second promoter initiates expression of the second foreign gene in the eukaryotic cell.

16. The method of claim **15**, wherein said virus is poxvirus.

17. The method of claim **16**, wherein said poxvirus is vaccinia.

18. The method of claim **15**, wherein said virus is vTF7-3 having A.T.C.C. Designation Number VR 2153.

19. The method of claim **15**, wherein said carrier is a DNA-based cytoplasmic virus.

20. The method of claim **19**, wherein said first gene is the T7 RNA polymerase gene.

21. The method of claim **15**, wherein said second promoter is a T7 promoter.

22. The method of claim **15**, wherein said carrier is a plasmid.

23. The method of claim 22, wherein said plasmid is pTF7CAT-1.

24. The method of claim 22, wherein said virus is poxvirus.

25. The method of claim 24, wherein said poxvirus is vaccinia.

26. The method of claim 22, wherein said plasmid is PTF7LZ-1.

27. The method of claim 19, wherein said carrier is vTF7LZ-1 having A.T.C.C. Designation Number VR 2152.

28. The method of claim 19, wherein said carrier is vTF7HB-1 having A.T.C.C. Designation Number VR 2154.

29. The method of claim 22, wherein said second foreign gene encodes HBsAg.

30. The method of claim 29, wherein said second promoter is a T7 promoter.

31. The method of claim 29, wherein said second foreign gene is lacZ.

32. The method of claim 29, wherein said second foreign gene is CAT.

33. A eukaryotic cell capable of expressing a foreign gene in the cytoplasm of the cell, comprising incorporated into said cell:

a DNA-based cytoplasmic virus including a first gene encoding a bacteriophage RNA polymerase selected from the group consisting of T7, SP6, GH1 and T3 RNA polymerase, said first gene being foreign to the virus and to the cell, and a first promoter sequence which initiates expression of said first gene; and

a carrier comprising a functional cistron comprising a second foreign gene including a second promoter sequence responsive to said bacteriophage RNA polymerase, such that upon recognition of said RNA polymerase, said second promoter initiates expression of the second foreign gene in the eukaryotic cell.

34. The cell of claim 33, wherein said virus is vaccinia.

35. The cell of claim 33, wherein said first gene is the T7 RNA polymerase gene.

36. The cell of claim 33, wherein said carrier is a plasmid.

37. The cell of claim 33, wherein said second promoter is a T7 promoter.

38. The cell of claim 33, wherein said cistron further includes a terminator sequence.

39. The cell of claim 33, wherein said carrier is a DNA-based cytoplasmic virus.

40. The cell of claim 39, wherein said carrier is vaccina.

41. The cell of claim 39, wherein said second promoter is a T7 promoter.

42. The cell of claim 39, wherein said cistron further includes a terminator sequence.

43. A DNA-based cytoplasmic virus containing a foreign gene encoding a bacteriophage RNA polymerase selected from the group consisting of T7, SP6, GH1 and T3 RNA polymerase.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,550,035

DATED : August 27, 1996

INVENTOR(S) : Moss, et. al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Title page, item [73] Assignee, the following text should be added: --; Government of the United States of America Secretary, Department of Health and Human Services, Rockville, M.D.--

Signed and Sealed this

Eleventh Day of February, 1997

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

EXHIBIT 2

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Specific Contacts between the Bacteriophage T3, T7, and SP6 RNA Polymerases and Their Promoters*

(Received for publication, July 23, 1990)

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The specificity and structural simplicity of the bacteriophage T3, T7, and SP6 RNA polymerases make these enzymes particularly well suited for studies of polymerase-promoter interactions. To understand the initial recognition process between the enzyme and its promoters, DNA fragments that carry phage promoters were chemically modified by three different methods: base methylation, phosphate ethylation, and base removal. The positions at which these modifications prevented or enhanced binding by the RNA polymerases were then determined. The results indicate that specific contacts within the major groove of the promoter between positions −5 and −12 are important for phage polymerase binding. Removal of individual bases from either strand of the initiation region (−5 to +3) resulted in enhanced binding of the polymerase, suggesting that disruption of the helix in this region may play a role in stabilization of the polymerase-promoter complexes.

The DNA-dependent RNA polymerases that are encoded by bacteriophages T7, T3, and SP6 are particularly well suited for studies of polymerase-promoter interactions. Each of the phage enzymes consists of a single species of protein of ≈100 kDa that is able to carry out all of the steps in the transcription process in the absence of any additional protein factors (for review, see Chamberlin and Ryan, 1983). The T7 RNA polymerase has recently been crystallized, indicating that detailed structural information will soon be available (Sousa *et al.*, 1989).

Despite a high degree of structural similarity (as judged by the conservation of their amino acid sequences) the phage RNA polymerases are exquisitely specific for their own promoter sequences. Each of the phage promoters is related to a common 23-base pair consensus sequence that extends from −17 to +6 (see Fig. 1). A core nucleotide sequence that extends from −7 to +1 is highly conserved throughout the three promoter types, suggesting that this region interacts with shared features of the polymerases in a common manner. The promoter sequences differ significantly in the region from −6 to −12, suggesting that the base pairs in this region are important for specific promoter recognition. Studies with synthetic phage promoters have shown that the primary determinants of promoter specificity for the T3 and T7 enzymes involve the base pairs at positions −10 and −11; substitution of T3 residues at these two positions in the T7 promoter sequence prevents recognition by the T7 polymerase and enables transcription by the T3 RNA polymerase (Klement *et al.*, 1990). A domain in the T3 and T7 RNA polymerases that is responsible for the recognition of these determinants has been localized, and preliminary crystallographic data suggest that this region of the polymerase is positioned within a putative DNA-binding cleft (Joho *et al.*, 1990).[1]

Considerable information is now available concerning polymerase-promoter contacts and the functional role of elements within the promoter. Studies with promoter mutants suggest that the promoter consists of a binding domain that extends from about −17 to −4 and an initiation domain that extends from about −4 to +6 (Chapman and Burgess, 1987; Schneider and Stormo, 1989; Klement *et al.*, 1990). Base substitutions in the binding domain have a strong effect upon polymerase binding but relatively little effect on the rate of initiation. In contrast, base substitutions in the initiation domain have little effect upon polymerase binding but decrease the rate of initiation.

Footprinting of T3 and T7 RNA polymerase-promoter complexes with DNase I and methidium propyl-EDTA-Fe(II) has shown that the polymerase protects a region from about −21 to −3 upon binding (Basu and Maitra, 1986; Ikeda and Richardson, 1986; Gunderson *et al.*, 1987). More recent experiments using the nonintercalating agent Fe(II)-EDTA provide greater resolution and have revealed two discrete protected regions located on one face of the DNA approximately one turn of the helix upstream from the start site (Muller *et al.*, 1989). Although the protected regions flank the major groove, no contact with residues within the major groove in the area that lies between these regions has been demonstrated.

After binding to the promoter, the polymerase melts open a small region of the promoter around the start site, as judged by a hyperchromic shift (Oakley *et al.*, 1979) and by the sensitivity of this region to attack by single-strand-specific endonucleases (Osterman and Coleman, 1981; Muller *et al.*, 1989). In the presence of limiting amounts of substrate (ribonucleoside triphosphates) the methidium propyl-EDTA-Fe(II) footprint changes (Ikeda and Richardson, 1986; Gunderson *et al.*, 1987). In the presence of GTP alone (which would permit the synthesis of a 3-nucleotide product) the

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[1] R. Sousa and B. C. Wang, personal communication.

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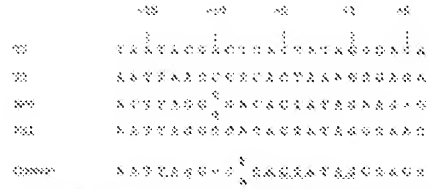

FIG. 1. **Comparison of the consensus sequences of four phage promoter types.** The consensus sequences of promoters for the T7 (Dunn and Studier, 1983), T3 (Bailey *et al.*, 1983), SP6 (Brown *et al.*, 1986), and K11 (Klein, 1986) RNA polymerases are presented. All of the phage promoters share a core sequence that extends from −7 to +1, suggesting that this region plays a common function in promoter function. The promoters diverge in the region from −8 to −12, suggesting that promoter-specific contacts are being made in this region. By convention, the sequence of the nontemplate strand is presented.

polymerase protects the region from −31 to +8. In the presence of GTP and ATP (which would permit the synthesis of a 6-nucleotide RNA product) the region from −21 to +11 is protected. These results suggest either that there is a conformational change in the DNA-polymerase complex during the early stages of binding and initiation (Ikeda and Richardson, 1986) or that there is protection of the DNA template as a consequence of movement of the polymerase along the DNA during these stages (Muller *et al.*, 1988).

Detailed structural information concerning specific contacts between the polymerase and promoter will become of considerable importance as crystallographic data begins to emerge. For this reason, a variety of approaches toward understanding polymerase binding will prove informative. In this work, we have refined the molecular model of the DNA surface that is contacted during the binding process through the use of methylation and ethylation interference techniques (Siebenlist and Gilbert, 1980). Our results demonstrate that specific residues in the major groove between positions −6 and −12 are important for polymerase binding. In addition, we also used missing contact analysis (Brunelle and Schleif, 1987) to identify bases whose removal effected promoter binding. Interestingly, elimination of bases from either strand of the initiation region (−6 to +8) resulted in enhanced binding of the RNA polymerase, suggesting that disruption of the helix in this region promotes local DNA unwinding and stabilizes the polymerase-promoter complexes.

The genes for the T3 and T7 RNA polymerases have previously been cloned and overexpressed, facilitating biochemical studies with these enzymes (Davanloo *et al.*, 1984; Morris *et al.*, 1986). In this report, we describe the cloning and expression of the SP6 RNA polymerase gene and a procedure for purification of the enzyme.

MATERIALS AND METHODS

Preparation of T3 and T7 RNA Polymerases—T7 and T3 RNA polymerases were purified from cultures of *Escherichia coli* BL21 carrying the plasmids pAR1219 (Davanloo *et al.*, 1984) or pCM36 (Morris *et al.*, 1986), as previously described. The polymerase preparations had specific activities of $\approx 3 \times 10^7$ units/mg.

Cloning, Expression, and Purification of the SP6 RNA Polymerase—The region of the SP6 RNA polymerase gene that extends from a *Hin*dIII site located 35 base pairs upstream of the start codon (the last 6 nucleotides of the recognition sequence for this enzyme are apparent in the sequence of Kotani *et al.*, (1987)) to a *Bcl*XI site that

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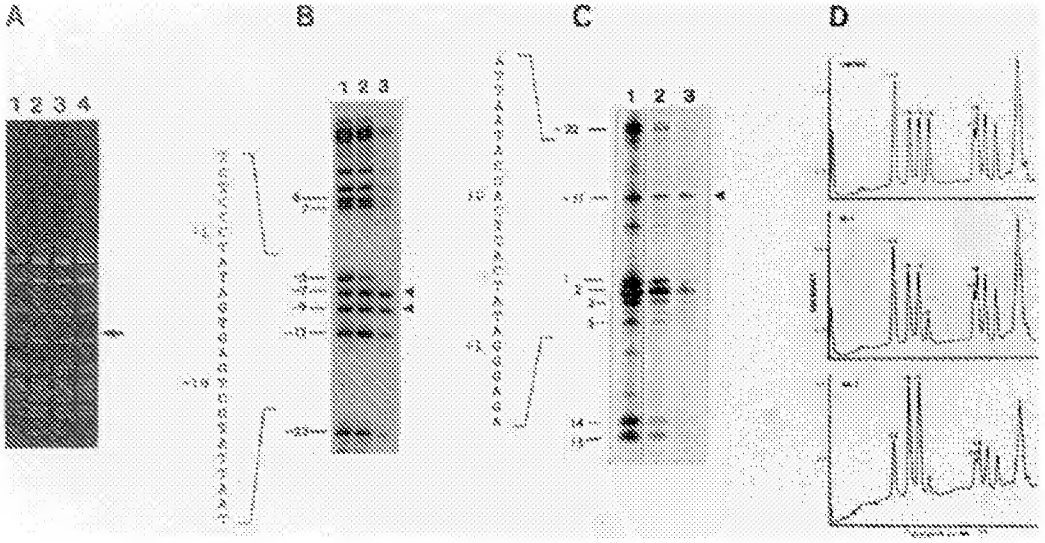

FIG. 2. **Methylation interference experiments with the T7 ϕ10 promoter.** *Panel A*, plasmid pAR436 (which contains the T7 ϕ10 promoter) was reacted with dimethyl sulfate and digested with *Bam*HI and *Eco*RI. The DNA fragments were incubated with increasing concentrations of T7 RNA polymerase and resolved by electrophoresis in a 6% polyacrylamide gel. Polymerase:promoter molar ratios were: *lane 1*, no polymerase; *lane 2*, 16:1; *lane 3*, 32:1; *lane 4*, 64:1. The *arrow* indicates the position of the fragment that contains the T7 ϕ10 promoter; binding of polymerase leads to the preferential disappearance of this band as the polymerase concentration is increased. *Panels B* and *C*, promoter-containing DNA fragments isolated from the binding gel were cleaved with piperidine before electrophoresis. *Panel A* (template strand) shows results obtained with the *Bam*HI-*Eco*RI fragment of pAR436 containing the T7 ϕ10 promoter. *Panel B* (nontemplate strand) shows results obtained with an *Xba*I-*Sph*I fragment of pAR436. The sequence of the promoter is given to the *left* of each panel. Polymerase:promoter ratios were: *lane 1*, no polymerase; *lane 2*, 32:1; *lane 3*, 64:1. *Lane 1* (no polymerase) represents a G ladder for the strands indicated. *Panel D*, densitometer scans of the autoradiograms presented in *panel B*. Note that methylation of G at position −5 on the template strand results in enhanced polymerase binding.

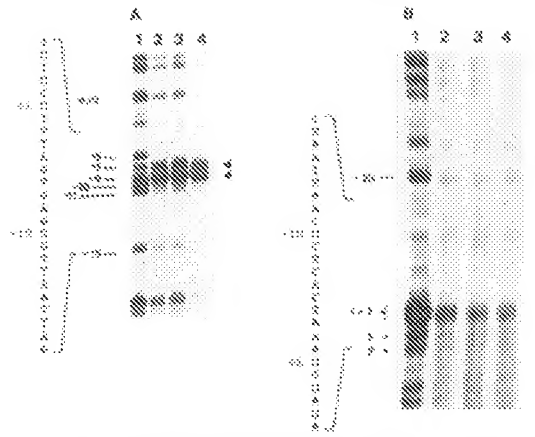

FIG. 3. **Methylation interference experiments with the T3 φ1.0 promoter.** Experiments were carried out as described in the legend to Fig. 2. *Panel A* (template strand) shows results obtained with an *Eco*RI-*Hin*dIII fragment of pIB30 that contains the T3 φ1.0 promoter. Polymerase:promoter ratios were: *lane 1*, no polymerase; *lane 2*, 8:1; *lane 3*, 16:1; *lane 4*, 32:1. *Panel B* (nontemplate strand) shows results obtained with a *Hin*dIII-*Eco*RI fragment of pIB30. Polymerase:promoter ratios were: *lane 1*, no polymerase; *lane 2*, 16:1; *lane 3*, 32:1; *lane 4*, 64:1. *Lane 1* (no polymerase) represents a G ladder for the strands indicated. *Arrows* indicate nucleotide positions where methylation results in interference with polymerase binding (−7 and −9 of the template strand). Note that, as in the case of the T7 promoter, methylation of the base at −8 of the template strand results in enhanced binding by the polymerase.

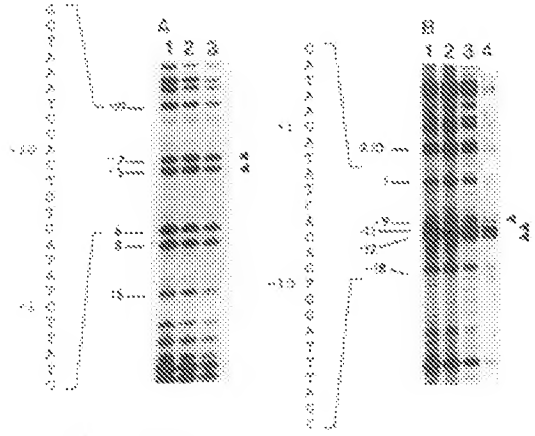

FIG. 4. **Methylation interference experiments with an SP6 promoter.** Experiments were carried out as described in the legend to Fig. 2. *Panel A* (template strand) shows results obtained with a *Hin*dIII-*Sph*I fragment of pSP6 that contains an SP6 promoter. Polymerase:promoter ratios were: *lane 1*, no polymerase; *lane 2*, 25:1; *lane 3*, 50:1. *Panel B* (nontemplate strand) shows results obtained with a *Hin*dIII-*Sph*I fragment of pSP6. Polymerase:promoter ratios were: *lane 1*, no polymerase; *lane 2*, 25:1; *lane 3*, 50:1; *lane 4*, 100:1. *Lane 1* (no polymerase) represents a G ladder for the strands indicated. *Arrows* indicate nucleotide positions where methylation results in interference with polymerase binding (−5 and −7 of the template strand; −9, −11, and −12 of the nontemplate strand).

lies 88 base pairs downstream from the termination codon (at nucleotide 2766) was cloned as a blunt-ended fragment under the control of the *lac*UV5 promoter in the plasmid pCM63 (Morris *et al.*, 1986), resulting in the plasmid pIB30.

Purification of the SP6 RNA polymerase was based on the method described by Goldberg and Dunn (1986) for purification of the T7 RNA polymerase. Cultures of BL21[pSR3] were propagated in Luria broth (Maniatis *et al.*, 1982) containing 50 μg of ampicillin/ml to an absorbance of 0.8 at 600 nm. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM, and after 4 h the cells were harvested by centrifugation. The cell pellet (9 g from 1 liter of culture) was washed once with 400 ml of HB (50 mM Tris·HCl, pH 8, 2 mM EDTA, 30 mM NaCl, 1 mM DTT[a]), resuspended in 24 ml of HB, and stored at −70° C. All subsequent steps were performed at 4 °C.

Frozen cells were thawed and the volume was adjusted to 100 ml of HB. Phenylmethylsulfonyl fluoride (0.1 mM), DTT (2 mM), and lysozyme (150 μg/ml) were added to the final concentrations indicated, and the mixture was incubated at 0 °C for 30 min. The cells were lysed by the addition of sodium deoxycholate (0.65%), $MgCl_2$ (15 mM), and DNase I (50 μg/ml, Sigma), and the lysate was incubated for 30 min with occasional stirring.

Ammonium sulfate was added to a final concentration of 0.2 M by the addition of 3.28 ml of a 5.5 M solution, and the mixture was stirred for 15 min. The sample was centrifuged at 15,000 rpm for 20 min in a Sorvall SS34 rotor. One-tenth volume of 5% polyethyleneimine (BDH Chemicals, Ltd.) adjusted to pH 8 with HCl was added to the supernatant with constant stirring over a 1-min period, and stirring was continued for a further 15 min. The sample was clarified by centrifugation at 15,000 rpm for 20 min. A further 1.25 volume of 5.5 M ammonium sulfate was added to the supernatant with constant stirring over 30 min for a final concentration of 2.5 M ammonium sulfate. After 30 min, the sample was centrifuged at 15,000 rpm for 15 min. The precipitate was gently taken up in 150 mM NaCl in buffer B (50 mM KPO_4, pH 7.7, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT). The solution was clarified by centrifugation at 15,000 rpm for 15 min and the sample was applied to a Bio-Gel P-60G column (2.5 × 85 cm, Bio-Rad). The column was eluted with buffer B containing 150 mM NaCl at a flow rate of 120 ml/h in 6-ml fractions. Void volume fractions were identified by spotting 40-μl fractions onto filter paper, staining with 0.02% Coomassie Blue in 25% isopropyl alcohol, 10% acetic acid, and destaining with 5% isopropyl alcohol, 10% acetic acid. Since the polymerase constitutes a large fraction of the total lysate protein, this method was used throughout the purification to identify peak fractions. Void volume fractions were pooled and applied to a DEAE-Sepharose column (1.5 × 15 cm, Pharmacia LKB Biotechnology Inc.). The column was washed with 60 ml of 150 mM NaCl in buffer B at a flow rate of 180 ml/h and then eluted with 300 mM NaCl in buffer B in 1.5-ml fractions. Peak fractions were identified by electrophoresis of aliquots in polyacrylamide gels in the presence of sodium dodecyl sulfate (Laemmli, 1970), followed by staining with Coomassie Blue. The pooled fractions were diluted with buffer B containing 95 mM NaCl to result in a final concentration of 200 mM NaCl and applied to a P11 phosphocellulose column (6.5 × 10 cm, Whatman). The column was washed with 20 ml of buffer B containing 200 mM NaCl at a flow rate of 8.5 ml/h and eluted with buffer B containing 500 mM NaCl at a flow rate of 8.5 ml/h. Peak fractions (6.5 ml each) were pooled and dialyzed overnight against 2 volumes of storage buffer (20 mM KPO_4, pH 7.7, 1 mM EDTA, 100 mM NaCl, 50% (v/v) glycerol, 1 mM DTT). Polymerase assays (Morris *et al.*, 1986) were carried out in a volume of 50 μl containing 2 μg of SP6 phage DNA. The final enzyme preparation had a specific activity of >10[6] units/mg of protein (Butler and Chamberlin, 1982) and is >95% pure as judged by polyacrylamide and electrophoresis (data not shown).

Preparation of Modified Promoter-containing DNA.—Plasmid pIB30 contains the T3 φ1.0 promoter (map position, 84.6%; Bailey *et al.*, 1983), pAR456 contains the T7 φ1.0 promoter (Studier and Rosenberg, 1981), and pSP65 (Promega Corp.) carries a class II SP6 promoter (Melton *et al.*, 1984; Brown *et al.*, 1986). All plasmids were propagated in *E. coli* BB101 (Maniatis *et al.*, 1982). Plasmids were digested with restriction enzymes, and the resulting fragments were radioactively end-labeled using either [α-^{32}P]NTPs and the large fragment of *E. coli* DNA polymerase (Pol1K) or [γ-^{32}P]ATP and polynucleotide kinase (Maniatis *et al.*, 1982). The DNA was subsequently treated with a second restriction enzyme to yield a mixture of fragments, one of which contained the promoter and was labeled at only one end.

Three types of modifications were used in our interference studies: base methylation, phosphate ethylation, and base removal. Methylation with dimethyl sulfate was performed as described by Siebenlist and Gilbert (1980). Methylation mixes containing 1 μg of DNA and

[a] The abbreviation used is: DTT, dithiothreitol.

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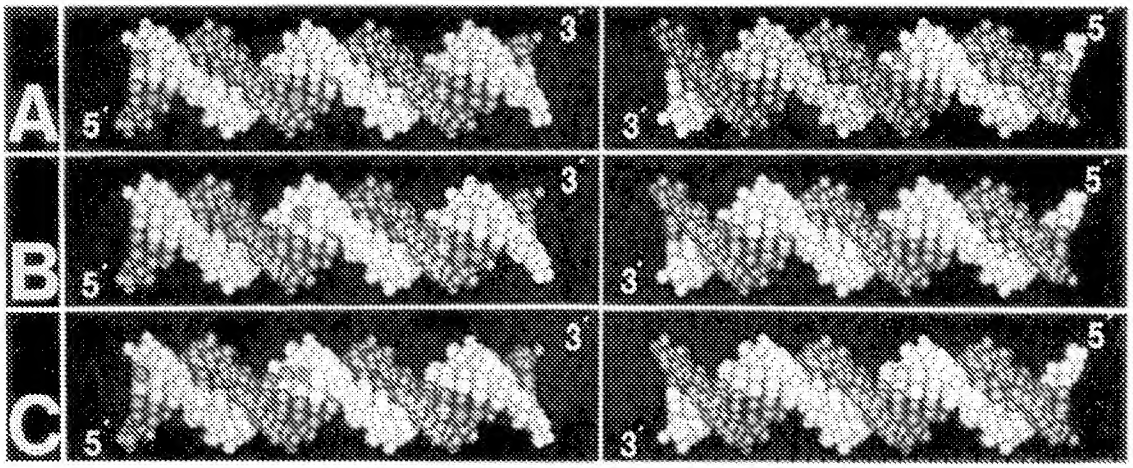

FIG. 5. **Summary of methylation interference data for the bacteriophage promoters.** *Panel A*, SP6 promoter; *panel B*, T7 promoter; *panel C*, T3 promoter. Template strands are *white*; nontemplate strands are *blue*. *Red* atoms indicate N7 positions of guanine residues where methylation results in binding interference. *Yellow* indicates enhancement of binding as a result of methylation. All other guanine N7s are *green*. A *white* cross indicates the N7 of the base at the +1 position. Computer graphics were performed with the program Promodeler 1 (New England BioGraphics). Sequences shown are those presented in Figs. 2–4.

1 μl of dimethyl sulfate in 283 μl of 10 mM sodium cacodylate, pH 8.0, were incubated for 30 s at 20 °C. The reactions were terminated and the DNA was purified as described. Unlabeled plasmid DNA containing no bacteriophage promoter sequences was used as a carrier for all precipitations in this study rather than tRNA, which can inhibit binding of the phage polymerases to DNA (Gunderson *et al.*, 1987).[2] Methylated DNA was stored frozen for up to 1 week at −20 °C prior to use.

Ethylations were performed as described by Nehlenlist and Gilbert (1980). Reaction mixes containing 1–5 μg of DNA in 500 μl of 10 mM sodium cacodylate, pH 8.0, and 200 μl of ethanol saturated with ethylnitrosourea were incubated for 36 min at 50 °C. Following purification by repeated ethanol precipitation, the ethylated DNA was used immediately in interference experiments.

Base removal was carried out as described by Brunelle and Schleif (1987) except for the use of plasmid DNA as carrier as described above. Under the conditions used, each DNA molecule sustained, on average, less than one depurination event, as evidenced by the presence of the majority of the DNA in the uncleaved position of the sequencing gel following piperidine cleavage.

Interference Experiments—Modified DNA was divided into 0.5-μg aliquots and incubated with different concentrations of RNA polymerase at room temperature for 20 min under conditions that allow binding but not initiation. Binding reactions contained 10 mM Tris·HCl, pH 7.9, 8 mM $MgCl_2$, 1 mM DTT, and 4% glycerol in a volume of 20 μl. Polymerase:promoter molar ratios ranged from 0:1 to 10:1, as indicated. Following incubation, the mixtures were loaded onto 15-cm, 5% polyacrylamide gels in a Tris borate EDTA buffer and electrophoresed at 200 V for 2–3 h (Maxam and Gilbert, 1980). Promoter-containing fragments that have modifications that interfere with promoter binding enter the gel and migrate normally. These fragments were cut out of the gel and eluted for subsequent chemical cleavage and analysis on sequencing gels to reveal the positions of the interfering modifications (Siebenlist and Gilbert, 1980). Gels were exposed to Kodak XAR5 film, and the autoradiograms were analyzed by densitometry using an LKB laser densitometer.

RESULTS

Methylation Interference—Plasmids that contain a phage promoter were digested with appropriate restriction enzymes, and the DNA fragments were end-labeled with ^{32}P. The DNA was then treated with dimethyl sulfate under conditions in which the N7 position of G residues is preferentially methylated. The modified DNA fragments were incubated with increasing concentrations of RNA polymerase and the enzyme/DNA mixtures were resolved by electrophoresis in 5% polyacrylamide gels (Fig. 2*A*). Under these conditions, restriction fragments that are bound by the polymerase migrate anomalously whereas unbound fragments migrate normally. As the polymerase:promoter ratio increases, the intensity of the band corresponding to the promoter-containing fragment decreases. The polymerase-promoter complexes are relatively unstable in this gel system and dissociate during the electrophoresis run; for this reason, discrete bands that would correspond to the polymerase-promoter complex are not observed (Ling *et al.*, 1989; Klement *et al.*, 1990). Promoter-containing fragments that remained unbound at high concentrations of RNA polymerase were eluted from the gel, treated with piperidine to cleave the DNA at the site of methylation, and analyzed by electrophoresis.

The effects of methylation of the template strand of the T7 φ10 promoter are shown in Fig. 2*B*. Bands that become enriched as the polymerase concentration is increased indicate positions where methylation of G residues interferes with binding. Clearly, methylation of G residues at positions −7 or −8 on the template strand resulted in interference of binding. An unexpected finding was that methylation of the G residue at position −5 resulted in an unusually marked decrease in the intensity of the corresponding gel band with increasing polymerase concentration. This result indicates that methylation at this position enhances polymerase binding.

With regard to contacts with the nontemplate strand, the data of Fig. 2*C* show that methylation of the G at position −11 interferes with binding.

We see no effect on binding by methylation of the G residues at positions +1 to +3 and +5 on the nontemplate strand or at −12 on the template strand. As noted above, footprinting experiments have shown that the T7 RNA polymerase extends only to about −4 in the absence of substrate (Ikeda and Richardson, 1986; Gunderson *et al.*, 1987). In light of this observation, it is not surprising that methylation of the G residues around the initiation site produced no inter-

[2] Y. Zhou and W. T. McAllister, unpublished observations.

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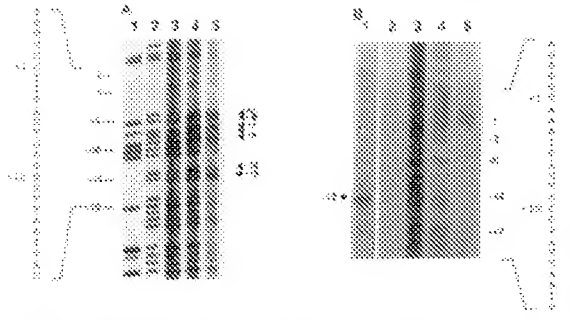

FIG. 6. **Ethylation interference experiments with the T3 ϕ10 promoter.** DNA was modified by treatment with ethylnitrosourea and incubated with T3 RNA polymerase. Promoter-containing DNA fragments were resolved by electrophoresis as described in the legend to Fig. 2. Eluted fragments were cleaved by treatment with heat in the presence of NaOH before electrophoresis. *Panel A* (template strand) shows results obtained with an *EcoRI-BstI* fragment of pBR8 that contains the T3 ϕ10 promoter. Polymerase:promoter ratios were *lane 3*, no polymerase; *lane 4*, 8:1; *lane 5*, 16:1. *Lanes 1* and *2* are markers that consist of the same fragments analyzed by standard sequencing methods (Maxam and Gilbert, 1980) using the G and G + A reactions, respectively. *Panel B* (nontemplate strand) shows results obtained with an *EcoRI-BstI* fragment of pBR8. Polymerase:promoter ratios were: *lanes 1* and *2*, 32:1; *lane 3*, no polymerase. *Lanes 4* and *5* are the G + A and G marker reactions, respectively. *Lane 1* has been deliberately overexposed to enhance detection of weak bands. *Arrows* indicate nucleotide positions where ethylation of the sugar-phosphate backbone resulted in decreased binding of T3 polymerase (−8 to −3, −9, −14, and −15 of the template strand; −12 of the nontemplate strand).

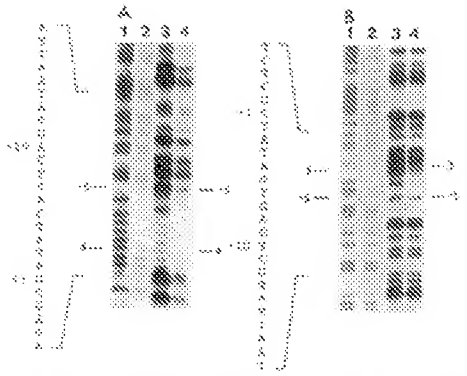

FIG. 7. **Base removal experiments with the T7 ϕ10 promoter.** DNA was modified by treatment with formic acid (G + A reaction) or hydrazine (C + T reaction) and incubated with T3 RNA polymerase. Promoter-containing DNA fragments were resolved by electrophoresis as described in the legend to Fig. 2. Eluted fragments were cleaved by treatment with piperidine before electrophoresis in sequencing gels. *Panel A* (template strand) shows results obtained with a *BamHI-DdeI* fragment of pAR436. *Panel B* (nontemplate strand) shows results obtained with an *NheI-SphI* fragment of pAR436. In both panels, *lanes 1* and *2* represent the G + A reactions, *lanes 3* and *4*, the T + C reactions. Polymerase:promoter ratios were: *lanes 1* and *3*, no polymerase; *lanes 2* and *4*, 100:1. Regions where base removal increases the efficiency of polymerase binding are indicated (−8 to +4 on the template strand; −6 to +6 on the nontemplate strand).

ference. Failure to observe interference at position −12 on the template strand indicates that the T7 RNA polymerase does not make a close approach to the *N*7 position of this base.

The usefulness of the methylation interference technique depends upon the presence of G residues at potential contact sites and is thus limited by the nature of acceptable promoter sequences. As more information can be obtained by looking at a variety of promoter types (see Fig. 1), we repeated the experiments above with the bacteriophage T3 and SP6 promoters and RNA polymerases.

Studies with the T3 RNA polymerase revealed an interference pattern similar to that observed in the T7 system (Fig. 3). Methylation at positions −7 and −9 on the template strand interfered with polymerase binding, and methylation at −8 resulted in enhanced binding. No interference was observed when the G residues at −10 and −11 on the template strand were methylated. The latter result was surprising in light of previous experiments in which we demonstrated the importance of the bases at −10 and −11 to the specificity and efficiency of utilization of these promoters (Klement et al., 1990). However, as noted above, methylation at position −11 of the nontemplate strand of the T7 promoter resulted in significant inhibition of binding by this enzyme. This result suggests that the polymerases may make asymmetric contacts within the major groove (see Fig. 5). This interpretation is consistent with data from Martin and Coleman (1987) who constructed T7 heteroduplex promoters having a T3 base on either the template or nontemplate strand at −10. In these experiments, it was found that the T7 RNA polymerase would tolerate the T3 base at −10 on the template strand but not on the nontemplate strand.

The importance of contacts with the nontemplate strand upstream of −8 is also seen in experiments with the bacteriophage SP6 promoter (Figs. 4 and 5). In this case, methylation of G residues at −8, −11, and −12 in the nontemplate strand resulted in interference. Interference was also observed after methylation at positions −7 and −5 of the template strand. Whereas binding of the T3 and T7 RNA polymerases to their promoters was enhanced when the −8 position of the template strand was methylated, methylation of the SP6 promoter at the −8 position interfered with the binding of this polymerase. Despite the differences in effect, these data indicate that all of the phage polymerases make a close approach to the *N*7 position of this base.

Ethylation Interference—Treatment of DNA with ethylnitrosourea results in the ethylation of phosphate groups along the backbone of the DNA (Sobeniast and Gilbert, 1980). The effects of these modifications on binding of the RNA polymerases were determined in a fashion similar to that described above. Following the binding reaction, promoter-containing restriction fragments that remained unbound in the presence of high concentrations of polymerase were eluted, and the end-labeled fragments were cleaved at the site of ethylation by heating in the presence of sodium hydroxide. The results of an ethylation interference experiment using the T3 promoter are shown in Fig. 6. Ethylation of the phosphates at −8 to −7, −9, −14, and −15 on the template strand and −12 on the nontemplate strand interfered with polymerase binding. These positions are located primarily on one face of the helix and coincide closely with the region that has been shown to be protected from cleavage by Fe(II)-EDTA (Muller *et al.*, 1988). The most marked interference was seen with ethylation of phosphates on either side of the major groove in the region where we have noted specific contacts to G residues and to a lesser extent on the distal side of the minor groove that lies upstream of this region. Similar results were obtained using the T7 ϕ10 promoter (see Fig. 8).

Base Removal Experiments—Individual bases within the DNA helix may be removed by treatment with formic acid or hydrazine (Maxam and Gilbert, 1980; Brunelle and Schleif,

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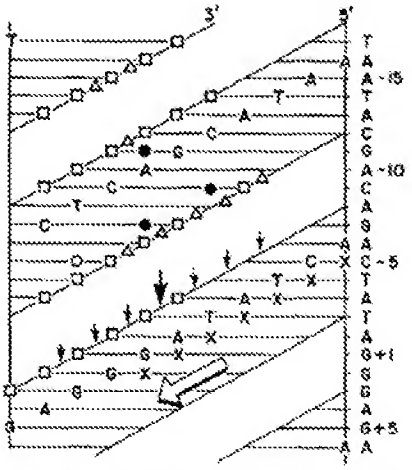

FIG. 8. **Topology of contacts between the T7 RNA polymerase and its promoter.** The results of experiments from a number of different laboratories are summarized. The sequence of the nontemplate strand of the T7 φ10 promoter from −17 to +6 is presented. ●, positions where methylation of G residues results in interference of binding; ○, positions where methylation leads to enhanced binding; □, ribose moieties that are protected from cleavage by Fe(II)-(EDTA) (Muller *et al.*, 1989); Δ, positions where ethylation of phosphates interferes with binding. The transcription start point and the direction of transcription is indicated by the *open arrow*. A region in which removal of individual bases leads to enhanced binding of the RNA polymerase is indicated in the major groove by ∞. The region of the nontemplate strand that is susceptible to cleavage by single strand-specific endonuclease (Osterman and Coleman, 1981) is indicated by *vertical arrows*; a site of particular sensitivity is indicated by the *large arrow*.

1987). The effects of these DNA modifications upon polymerase binding were explored using the T7 φ10 promoter. As can be seen from the data in Fig. 7, the removal of any individual base along the promoter was not sufficient to prevent polymerase binding. An unexpected result was that polymerase binding was *enhanced* when bases in the region from −3 to +3 on either strand were removed, indicating that removal of the bases in this region increases the binding affinity of the RNA polymerase for the DNA. The region where removal of bases leads to increased affinity corresponds closely with the region that is melted open upon polymerase binding (Osterman and Coleman, 1981; Muller *et al.*, 1989). We propose that the RNA polymerase melts this region during or after binding, and that destabilization of the helix as a consequence of removal of bases (Ullman and McCarthy, 1973) helps to promote the formation of an "open" complex.

DISCUSSION

In this report, we have probed the contacts of the phage RNA polymerases with their promoters by modifying the DNA with various chemical reagents and examining the effects of these modifications on polymerase binding. Three types of modifications were utilized: methylation, in which the N7 position of guanine is methylated through the action of dimethyl sulfate; ethylation, in which the phosphate groups in the sugar-phosphate backbone are ethylated by the action of ethylnitrosourea; and base removal, in which individual bases are removed from the helical structure by limited treatment of the DNA with formic acid (depurination) or hydrazine (depyrimidinization). The results of these experiments and those of previous investigators are summarized in Fig. 8.

The data are consistent with the notion that the phage polymerases contact the promoter primarily on one face of the DNA helix and make numerous specific contacts in the region from −5 to −11. The results of the methylation interference studies suggest that multiple contacts with residues in the major groove are made in this region. If we assume that all three phage RNA polymerases contact their promoters in a similar fashion and that the DNA in this region is in the B conformation, our data suggest that the contacts in the major groove may be asymmetric, as the enzymes appear to be more susceptible to the presence of blocking groups on the nontemplate strand upstream from −9 and on the template strand downstream from position −9 (see Fig. 5).

The observation that the polymerases make close approaches to the base pairs at −11, −10, and −5 would appear to be incongruous with a "one-face" approach of the polymerase to the promoter because the N7 of the base at −5 would lie on the "back" face of the helix relative to the bases at −10 and −11. However, previous data suggest that the region from −6 to +2 is not in a B form in the polymerase-promoter complex, but is melted open (Osterman and Coleman, 1981; Muller *et al.*, 1989). It may thus be possible for the polymerase to contact the N7 position of this base without wrapping around the helix.

In previous work, we also implicated the base pair at −15 in specific promoter recognition (Klement *et al.*, 1990). The major groove contacts at this position would also be located on the back side of the DNA helix relative to base pairs at −10 and −11. Preliminary crystallographic data suggest that the putative DNA-binding cleft of the T7 RNA polymerase is large enough to accommodate the helix and would allow contacts on both sides of the promoter structure.[4]

It is perhaps surprising that removal of bases in the binding region (−11 to − 5) did not result in decreased polymerase affinity. One interpretation of this result is that the phage polymerases make multiple contacts in the binding region and that disruption of a single (or a few) contacts does not prevent binding. This is in contrast to interference experiments in which the insertion of a steric blocking group (by methylation or ethylation) disrupts multiple contacts. We cannot rule out the possibility that the loss of one contact in this region weakens the binding affinity of the polymerase for the DNA but that under our assay conditions the loss is not sufficient to prevent retardation of the restriction fragment in the gel binding assay.

The conditions used for methylation in our experiments (very short exposure to dimethyl sulfate) result predominantly in methylation at the N7 position of G residues, which lies in the major groove. Less extensive methylation of A residues at the N3 position in the minor groove may also occur under these conditions (Maxam and Gilbert, 1980), and in some of our experiments, we have observed limited cleavage at A residues (see, for example, Fig. 4*B*). Nevertheless, in none of the experiments that we performed did we see evidence for interference of binding as a result of methylation at A residues. This may reflect the low incidence of A methylation in our samples. Alternatively, our failure to observe interference may indicate that the polymerase is not making specific contacts with the N3 position of A residues. As recently observed by Yang and Nash (1989), DNA-binding proteins seem to fall into two categories. The binding of most DNA-binding proteins is preferentially inhibited by methylation of G residues, presumably reflecting the importance of contacts in the major groove. The binding of proteins in the second group (such as integration host factor) is preferentially inhib-

[4] R. Sousa and B. C. Wang, personal communication.

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ited by methylation at A residues, reflecting the importance of minor groove contacts. Our results suggest that the phage polymerases fall into the former category. These results are seemingly in contrast to earlier work by Stahl and Chamberlin (1978) in which the nontemplate strand of a T7 promoter was repaired by the action of DNA polymerase in the presence of various base analogs that would introduce perturbations into either the major or minor grooves. These experiments demonstrated that some modifications, such as replacement of all adenine residues with 2,6-diaminopurine or all guanine residues with hypoxanthine (both of which affect the minor groove) prevented utilization of the modified promoter. We cannot exclude the possibility that specific minor groove contacts that we did not probe are important for polymerase binding. An alternative explanation for the observations by Stahl and Chamberlin is that contacts with minor groove elements are important during the initiation step, perhaps within the region that is melted open during initiation. As we have not measured the effects of base modifications on initiation in our experiments, we cannot exclude this possibility.

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EXHIBIT 3

Proc. Natl. Acad. Sci. USA
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Biochemistry

RNA polymerase specificity of mRNA production and enhancer action

(RNA processing/enhancer selectivity/transient expression/RNA polymerase I promoter/chloramphenicol acetyltransferase gene)

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ABSTRACT To examine the RNA polymerase (EC 2.7.7.6) specificity of RNA maturation/utilization and transcriptional enhancement, we constructed a chimeric plasmid (pPolI-CAT) in which a promoter for mouse rRNA gene transcription was placed adjacent the coding sequences for chloramphenicol acetyltransferase (CAT; EC 2.3.1.28). A number of other constructs, including plasmids also containing a murine sarcoma virus enhancer or lacking any natural eukaryotic promoter sequences, were also prepared. In apparent agreement with earlier conclusions that an RNA polymerase I transcript can act as a messenger RNA, transient transfection of mouse L cells with pPolI-CAT yielded both high levels of transcription from the RNA polymerase I promoter and enzymatically active CAT protein. However, further examination revealed that CAT protein is not translated from RNA that begins at the normal rRNA transcription initiation site. Polysomal RNA is devoid of such RNA and instead consists of CAT-encoding transcripts that begin elsewhere in the mouse ribosomal DNA (rDNA) region. Since transcription of these aberrant RNAs is stimulated by the addition of a murine sarcoma virus enhancer segment, they are probably transcribed by RNA polymerase II. Transcripts that map to the authentic rRNA start site are not similarly enhanced. Moreover, unlike the RNAs deriving from the rRNA initiation site, these aberrant RNAs are more stable and the level of translatable CAT transcripts is suppressed by inclusion of larger segments of the rDNA promoter regions. Fortuitously initiated mRNAs are also formed in the absence of any natural eukaryotic promoter sequence. From these data we conclude (*i*) that there is no evidence that normal RNA polymerase I transcription yields functional mRNA and (*ii*) that transcriptional enhancement appears to be RNA polymerase specific.

Precisely how nuclear transcripts whose synthesis is catalyzed by RNA polymerase I, II, or III (DNA-directed RNA polymerase, EC 2.7.7.6) are directed through the appropriate processing/transport/utilization pathway remains largely unresolved. *A priori*, the choice of the maturation pathway could be specified solely by the primary sequence of the transcript. Alternatively, it could be dependent upon the polymerase that catalyzes the transcription either through subnuclear compartmentalization or cotranscriptional attachment of processing complexes. Certain aspects of this general question have been approached by Green *et al.* (1), who followed the fate of RNA synthesized *in vitro* that was injected into *Xenopus* oocytes. These authors showed that the injected RNA was spliced accurately, albeit quite inefficiently. This finding, along with the development of *in vitro* systems that support splicing (2–4), polyadenylylation (5), and 3′-end cleavage (6, 7) of purified RNAs normally transcribed by RNA polymerase II, has demonstrated that there is not an obligatory coupling of processing of mRNA precursors to concomitant synthesis by a polymerase II transcription complex.

Other studies have begun to address the companion questions of whether ribonucleoprotein particles that arise from transcription by RNA polymerase I or III are appropriate substrates for mRNA processing and transport pathways, and moreover, whether such RNAs are competent to direct protein synthesis. Carlson and Ross (8, 9) have reported that alternative transcripts of the human and mouse β-globin genes, evidently produced by RNA polymerase III, appear to be spliced normally. These unusual globin transcripts are either polyadenylylated (8, 9) or "transspliced" onto normal globin RNA. Moreover, from microinjection and transfection studies, Grummt and co-workers have reported that the promoter of the mouse rRNA gene can direct transcription of a juxtaposed simian virus 40 (SV40) tumor (T) antigen (10) or chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) (11) coding region and that these RNAs are subsequently translated into the corresponding polypeptides. Since a substantial proportion of the transcripts do initiate at the normal rRNA initiation site, Grummt and coworkers concluded that normal polymerase I transcription can yield a functional translatable mRNA (11).

We have further investigated whether functional polypeptides can be encoded by a hybrid gene transcribed by RNA polymerase I. To this end, we have cloned the mouse RNA promoter region juxtaposed to the coding region of the CAT gene (12). After expression in transiently transfected mammalian tissue culture cells, the resultant CAT mRNA-containing transcripts were characterized by S1 nuclease and primer extension analyses, and production of functional CAT protein was determined by enzymatic assays. We find that synthesis of CAT protein is indeed directed by these constructs, as originally reported by Grummt and Skinner (11). However, none of the polysomal RNAs begins at the normal rRNA initiation site. Instead, the 5′ terminus of this translatable RNA maps to another site within the rRNA sequence. Inasmuch as the levels of this aberrant RNA and its translation product are increased by the presence in *cis* of a murine sarcoma virus (MSV) enhancer sequence, we suggest that the translatable CAT RNA may arise from a fortuitous polymerase II start in the rRNA initiation region. RNAs deriving from the authentic mouse rRNA start site are indeed detected, but they are not found on polysomes, are not increased in amount by the presence of an MSV enhancer, and are very short lived.

MATERIALS AND METHODS

Plasmids. The constructs (Fig. 1) were formed from pSV2-CAT (12), kindly provided by George Khoury. For pSV2′-CAT, the *Pvu* II site at the upstream boundary of the SV40

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; CAT, chloramphenicol transacetylase; MSV, murine sarcoma virus; rDNA, DNA encoding rRNA and the flanking spacer regions; bp, base pair(s); kb, kilobase(s).

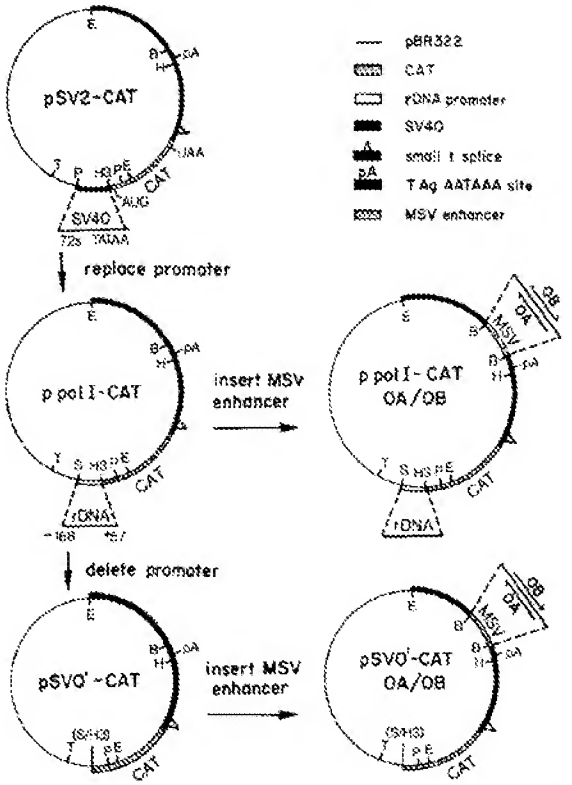

FIG. 1. Construction of hybrid CAT gene plasmids. As detailed in *Materials and Methods*, the SV40 promoter/enhancer region of pSV2-CAT (12) was replaced with the promoter region for mouse rRNA gene transcription [nucleotides −168 to +57 (13)] to yield pPolI-CAT, or the SV40 control region was deleted entirely to yield pSV0'-CAT. The translation initiation signals for the CAT gene are 3' to the *Hin*dIII site and are retained in all constructs. An MSV enhancer region was also inserted in both orientations (OA and OB) in pPolI-CAT and pSV0'-CAT. Restriction endonuclease sites: P, *Pvu* II; S, *Sal* I; H3, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; T, *Tth* HI I; R, *Hpa* I. Small t, SV40 small tumor antigen; 72s, SV40 72-bp repeats; rDNA, DNA encoding rRNA and the flanking spacer regions.

promoter/enhancer region was converted to a *Sal* I site by partial *Pvu* II digestion and ligation to *Sal* I linkers. This plasmid was used for subsequent constructs and as a "divergence probe" for S1 nuclease analysis of pSV2-CAT RNA. To form pPolI-CAT, the mouse rDNA plasmid p5'Sal-Pvu (14) was cleaved sequentially with *Sal* I (rDNA position −168) and *Taq* I (rDNA position +57). Next, pSV2'-CAT was cleaved with *Sal* I and *Hin*dIII to remove the entire SV40 promoter/enhancer region. The 235-base-pair (bp) rDNA promoter fragment (*Sal–Taq* fragment) and the CAT/vector (*Sal–Hin*dIII fragment) were isolated and joined by using *Hin*dIII–*Taq* converters to form pPolI-CAT. The CAT/vector fragment was also circularized by itself after treatment with the Klenow fragment of DNA polymerase to blunt the ends, forming the promoterless pSV0'-CAT. (Note that pSV0'-CAT is strictly homologous to pSV2-CAT and pPolI-CAT, while the standardly used pSV0-CAT (12) has different pBR322 sequences abutting the CAT region.) A 398-bp fragment of the MSV long terminal repeat (LTR) containing the 72/73-bp MSV enhancer repeats (ref. 15, kindly provided by G. Khoury) was inserted into the unique *Bam*HI site of pPolI-CAT and pSV0'-CAT in both orientations, termed OA (natural orientation) and OB (reverse orientation). Finally, to form p-2kbPolI-CAT a 1.8-kilobase (kb) *Sal* I fragment of mouse rDNA extending from position ≈−2 kb to −168 (16) was inserted at its natural position relative to the rDNA promoter at the unique *Sal* I site in pPolI-CAT.

Transfections and CAT Assay. Mouse L cells and Chinese hamster ovary (CHO) cells were transfected as described by using DEAE-dextran coupled with a dimethyl sulfoxide shock (17). Extracts were prepared 24 hr after transfection and assayed for CAT activity as described (12, 17).

RNA Analyses. RNA was prepared 24 hr after transfection by using the guanidinium isothiocyanate/cesium chloride method (18). Alternatively, polysomes were prepared (19) prior to RNA isolation. For S1 nuclease analysis, single-stranded DNA probes were prepared as described (20) and end labeled at the *Pvu* II site within the CAT gene. Probe (0.2 pmol) was hybridized to 10 μg of cellular RNA in 0.3 M NaCl/2 mM EDTA/100 mM Tris·HCl, pH 7.6, at 65°C for 12 hr, and single-stranded regions were digested with S1 nuclease (20). Alternatively, a primer (the 5'-end-labeled coding strand of the 27-bp *Hin*dIII–*Dde* I fragment at the 5' end of the CAT coding region) was hybridized to the RNA and extended by using reverse transcriptase as described (21). The resultant radioactive fragments were denatured, fractionated on 6% polyacrylamide sequencing gels, and visualized by autoradiography (20).

RESULTS

To examine whether RNA whose synthesis is catalyzed by RNA polymerase I can direct translation of a functional protein, we have utilized the hybrid plasmids displayed in Fig. 1. These constructs are based on pSV2-CAT of Gorman *et al.* (12), in which the bacterial CAT gene is inserted between the SV40 early transcription initiation segment and the SV40 small tumor antigen splice and polyadenylylation regions. To form a CAT gene transcribed under direction of a polymerase I promoter, we have replaced the entire SV40 promoter/enhancer region of pSV2-CAT with sequences from the mouse rRNA promoter region. The resultant pPolI-CAT contains rDNA sequence −168 to +57 (where +1 is the transcription start site) that includes the ≈150-bp polymerase I promoter defined *in vitro* (13). A second plasmid, pSV0'-CAT, which is strictly analogous to pPolI-CAT and pSV2-CAT but lacks all natural eukaryotic promoter regions, was constructed from pPolI-CAT by excision of the rDNA sequences. Derivatives of pPolI-CAT and pSV0'CAT into which an MSV enhancer segment was inserted in the natural (OA) or reverse (OB) orientation 3' to the CAT gene were also formed (Fig. 1), yielding pPolI-CAT-MSV(OA/OB) and pSV0'-CAT-MSV(OA/OB). A final plasmid (p-2kbPolI-CAT) containing an additional 1.8 kb of upstream rDNA sequence (residues ≈−2kb to +57) was also constructed from pPolI-CAT (not shown).

These plasmids were introduced into mouse L cells by transient transfection using DEAE-dextran and a dimethyl sulfoxide shock (17). Extracts prepared from the cells 24 hr after transfection were assayed for CAT activity (12) (Fig. 2). Consistent with an earlier report involving a similar construct (11), pPolI-CAT directs production of CAT enzymatic activity (Fig. 2, lane 4). Indeed, the CAT activity from pPolI-CAT is substantially greater than from the analogous "promoterless" construct, pSV0'-CAT, which yields a small but detectable level of CAT activity (lane 7). Strikingly, the presence of the MSV enhancer in pPolI-CAT results in markedly increased CAT activity (lanes 5 and 6), demonstrating that translatable RNAs from pPolI-CAT are elevated by this polymerase II enhancer segment. The level of CAT expression from pPolI-CAT-MSV(OA/OB) is comparable to that from pSV2-CAT (lane 10). However, the presence of the MSV enhancer also substantially raises the level of CAT expression from the promoterless constructs pSV0'-CAT-MSV(OA/OB) (lanes 8 and 9).

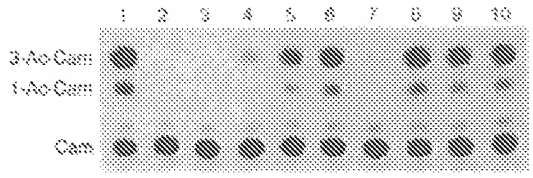

FIG. 2. Assay of CAT enzymatic activity from transfected L cells. L cells were transfected with the various plasmids and 40% of the extract prepared from a 60-mm dish was assessed for CAT activity. Unreacted chloramphenicol (Cam) and its 1- and 3-acetate derivatives were separated chromatographically. Lane 1, 10^{-2} unit of CAT enzyme (P-L Biochemicals); lane 2, extract from untransfected L cells; lanes 3–10, RNA from L cells transfected with p-2kbPolI-CAT (lane 3), pPolI-CAT (lane 4), pPolI-CAT-MSV(OA) (lane 5), pPolI-CAT-MSV(OB) (lane 6), pSV0'-CAT (lane 7), pSV0'-CAT-MSV(OA) (lane 8), pSV0'-CAT-MSV(OB) (lane 9), or pSV2-CAT (lane 10).

To directly assess expression of these plasmids in transfected L cells, we sought to analyze their transcripts. Unfortunately, S1 nuclease analysis cannot be used to characterize the transcripts of pPolI-CAT in mouse cells (20). This is because the primary rRNA transcript endogenous in the mouse L cells forms an S1-resistant trimolecular hybrid with pPolI-CAT probe DNA and pPolI-CAT-derived RNA, preventing detection of the actual initiation site of the pPolI-CAT RNA. Consequently, we have analyzed RNAs from in the transfected L cells by primer extension. A 27-nucleotide end-labeled primer complementary to the 5' end of the CAT sequence (extending from 62 to 88 nucleotides 3' to the natural rRNA start site of pPolI-CAT) was prepared and annealed with the RNA. After extension by reverse transcriptase, the products were analyzed by electrophoresis under denaturing conditions (Fig. 3). The major extension product obtained with RNA from cells transfected with pPolI-CAT (lane 3) co-migrates with the 88-nucleotide extension product obtained with RNA faithfully initiated on this plasmid in an *in vitro* transcription reaction (lane 2). Thus, transcription initiates at position +1 of the transfected pPolI-CAT, and the amount of this RNA compares favorably

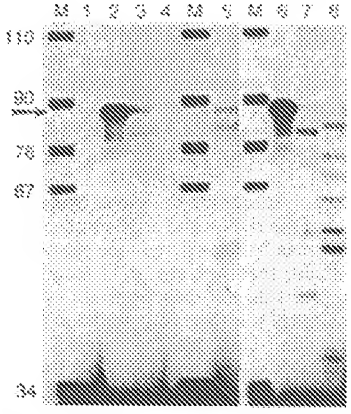

FIG. 3. Primer extension analysis of RNAs from transfected L cells. A 27-nucleotide primer from the 5' end of the CAT gene was hybridized to 20 μg of transfected RNA cell and the hybrids were extended by using reverse transcriptase. Lane 1, untransfected L cell RNA; lane 2, RNA transcribed *in vitro* from pPolI-CAT; lanes 3–5, RNA from L cells transfected with pPolI-CAT (lane 3), pSV0'-CAT (lane 4), or pSV2-CAT (lane 5); lane 6, RNA transcribed *in vitro* from pPolI-CAT-MSV(OB); lanes 7 and 8, RNA transcribed from L cells transfected with pPolI-CAT-MSV(OB) (lane 7) or pSV0'-CAT-MSV(OB) (lane 8). M, markers, with numbers of nucleotides indicated on the left.

to that obtained from cells transfected with pSV2-CAT (lane 5). However, since extension products of many other lengths are also observed in lane 3, it appears that transcripts in cells transfected with pPolI-CAT also originate from other than the rDNA initiation site.

RNA isolated from cells transfected with pSV0'-CAT directs production of only a relatively low level of extension product (Fig. 3, lane 4). The amount of this RNA is, however, increased by the presence of the MSV enhancer on the transfected pSV0'-CAT (lane 8), consistent with the CAT enzymatic assays (Fig. 2). This confirms that fortuitous initiation sites are present in pSV0'-CAT plasmid sequences and suggests that these direct synthesis of a limited amount of RNA that in turn is translated into CAT protein. Moreover, this fortuitously initiated translatable RNA is elevated by the presence of the MSV enhancer even when it is ≈2 kb away from the CAT gene.

In contrast, the MSV enhancer does not increase the level of RNA initiated accurately at rDNA residue +1 of pPolI-CAT (lanes 3 and 7, Fig. 3). Since the enhancer-containing plasmid pPolI-CAT-MSV(OA/OB) directed synthesis of considerably more CAT enzymatic activity than did pPolI-CAT (see Fig. 2), this suggests that polymerase I-derived transcripts do not yield translatable RNAs. However, since the MSV enhancer resulted in elevated levels of RNAs that initiated at sites other than rDNA position +1 in pPolI-CAT-MSV(OB) (Fig. 3, lane 7), it appears that RNA from pPolI-CAT that directs CAT protein synthesis does not initiate at the natural rDNA start site.

To further investigate which RNA species does direct CAT protein synthesis, polysomal RNA was isolated from cells transfected with pPolI-CAT, pPolI-CAT-MSV(OB), or pSV2-CAT, and the 5' ends of the resultant RNAs were identified. [Since polysomes do not contain RNA bearing the 5' end of the primary transcript of the cellular rRNA genes, polysomal RNA can be analyzed by S1 nuclease mapping without the complication of trimolecular hybrids (ref. 20; see above).] Fig. 4*A* shows an S1 nuclease analysis of this RNA. As expected, the 5' end of polysomal RNA from cells transfected with pSV2-CAT (lane 3) maps to the series of start sites known to be utilized by the SV40 early promoter region (22). Control RNA transcribed *in vitro* by polymerase I from pPolI-CAT in an S-100 transcription reaction similarly begins at the site expected for correct rRNA initiation (lane 4) and yields an ≈207-nucleotide protected fragment. (Since the SV40 and the rDNA promoters contribute approximately equal lengths of transcribed regions to pSV2-CAT and pPolI-CAT, respectively, their transcripts yield similar lengths of S1 nuclease or primer extension products.) Strikingly, however, no polysomal RNA from transfected pPolI-CAT (lane 1) or pPolI-CAT-MSV-OB (lane 2) begins at this normal start site. Instead, there are two other classes of polysomal pPolI-CAT-derived transcripts, both more prevalent in cells transfected with pPolI-CAT-MSV(OB) (lane 2) than in cells transfected with pPolI-CAT (lane 1). The 5' terminus of one of these RNAs maps to approximately position +32 of the rRNA gene; it corresponds to a relatively minor band of ≈55 nucleotides in the primer extension analysis of whole cell RNA (Fig. 3). The other class of RNA, detected as a 375-nucleotide protected "divergence band" (Fig. 4*A*, lanes 1 and 2), is transcribed through the rDNA promoter region from an initiation site or sites 5' to the rDNA sequences. We thus conclude that CAT enzymatic activity resulting from transfection of L cells with pPolI-CAT and pPolI-CAT-MSV does not derive from correct initiation by polymerase I at rDNA position +1, but rather it derives from aberrantly initiated RNAs.

To extend this result, we transfected CHO cells with the various CAT plasmids. Fig. 5 displays the resultant CAT enzymatic activity. CAT activity is indeed obtained with

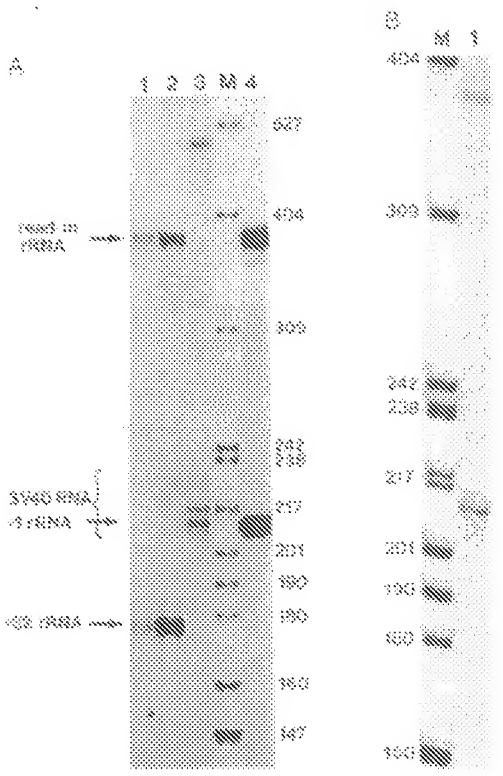

FIG. 4. S1 nuclease protection analysis of RNAs derived from transfected cells. (*A*) Analysis of polysomal RNA from L cells. Polysomal RNA was isolated from L cells transfected with pPolI-CAT (lane 1), pPolI-CAT-MSV(OB) (lane 2), or pSV2-CAT (lane 3). Lane 4, RNA transcribed from pPolI-CAT *in vitro* was analyzed. The probe for the pPolI-CAT RNAs (lanes 1, 2, 4) contains an insertion 5′ to the RNA polymerase I promoter region (375 nucleotides upstream of the labeled terminus). Correctly initiated RNA will thus yield a 207-nucleotide band protected from S1 nuclease, while any RNA that reads into this promoter region from upstream will yield a discrete 375-nucleotide band. The pSV2-CAT RNA was hybridized to an analogous "divergence" probe from pSV2′-CAT; correctly initiated RNAs will yield a series of bands of ≈205–225 nucleotides, while upstream initiated RNA will yield an ≈500-nucleotide "divergence band." M, markers, with numbers of nucleotides indicated on the right. (*B*) Analysis of RNA from CHO cells. RNA from CHO cells transfected with p-2kbPolI-CAT was mapped by S1 nuclease analysis as in *A*. RNA initiating at the correct rRNA transcription start site will yield a 207-nucleotide protected fragment, whereas RNAs that initiate 5′ to the rDNA sequences and transcribe into the rDNA sequences are visualized in a "divergence" band of 375 nucleotides.

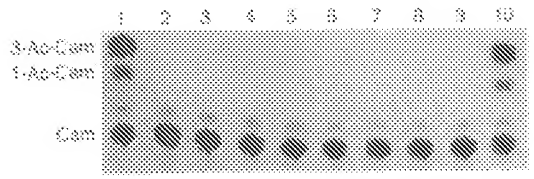

FIG. 5. Assay of CAT enzymatic activity from transfected CHO cells. CHO cells were transfected with the plasmids indicated below and the extract prepared from a 60-mm dish was assessed for CAT activity (see Fig. 2). Lane 1, 10^{-2} unit of CAT enzyme (P-L Biochemicals); lane 2, RNA from untransfected CHO cells; lanes 3–10, extract from CHO cells transfected with p-2kbPolI-CAT (lane 3), pPolI-CAT (lane 4), pPolI-CAT-MSV(OA) (lane 5), pPolI-CAT-MSV(OB) (lane 6), pSV0′-CAT (lane 7), pSV0′-CAT-MSV(OA) (lane 8), pSV0′-CAT-MSV(OB) (lane 9), or pSV2-CAT (lane 10).

pSV2-CAT (lane 10), demonstrating that CHO cells can be successfully transfected by this protocol. In contrast to the results found with the L cells in Fig. 2, however, no CAT activity is detectable in cells transfected with pPolI-CAT (lane 4) or its MSV enhancer-containing derivatives, pPolI-CAT-MSV(OA/OB) (lanes 5 and 6). Nor was CAT activity observed in pSV0′-CAT (lane 7) or pSV0′-CAT-MSV(OA/OB) (lanes 8 and 9) transfectants.

To test whether the lack of CAT activity in CHO cells transfected with rDNA-promoted CAT genes was due to an inability of the mouse RNA polymerase I promoter to function in hamster cells, we examined the RNA obtained from these transfected cells by S1 nuclease mapping. As seen in Fig. 4*B*, the mouse polymerase I promoter indeed directs synthesis of transcripts that initiate at position +1 of the rDNA promoter region. These results provide additional support for the conclusion that the translatable RNAs directed by pPolI-CAT do not derive from accurate initiation by RNA polymerase I.

DISCUSSION

The experiments described in this paper explore whether the rDNA promoter can direct the transcription of a gene that is normally transcribed by RNA polymerase II, and if so, whether the resultant RNA is a suitable substrate for the maturation pathway that leads to translatable RNAs characteristic of polymerase II-directed transcripts. Interest in these questions derives principally from two sources. First, an answer would serve to define the requisite specificity of the mRNA maturation pathway: is splicing/polyadenylylation/translation linked *in vivo* to an RNA polymerase II transcription complex, or alternatively, are RNAs segregated into appropriate processing and utilization pathways solely on the basis of a property inherent to the RNA transcript itself? Second, since the rRNA promoter is among the most active known transcriptional initiators it could potentially serve as a very useful promoter for active expression of a desired gene after transfection into growing cells.

The question of whether RNA polymerase I-directed transcripts can yield translatable RNAs has been addressed in two previous reports (10, 11), which concluded that RNAs that initiate at the rRNA start site and read into a T-antigen- or CAT-encoding region can indeed be translated into active protein. In accordance with these findings, using an analogous polymerase I promoter/CAT construct (pPolI-CAT) transiently transfected into mouse L cells, we do indeed observe the production of CAT enzymatic activity (Fig. 2) as well as of RNA transcripts that initiate correctly at position +1 of the rDNA (Fig. 3). However, the CAT-encoding RNAs on translating polysomes are devoid of transcripts that begin at position +1 of the rDNA and are composed only of species that initiate aberrantly elsewhere in the plasmid (Fig. 4*A*). We therefore conclude that the transcripts accurately initiated by RNA polymerase I do not direct protein synthesis.

The major class of the aberrantly initiated polysomal RNAs derived from pPolI-CAT and pPolI-CAT-MSV begins at approximately rDNA position +32. Since the normal ATG of the CAT sequence is the first ATG encountered in this RNA, it is likely that it is translated into CAT protein. Synthesis of a second class of polysomal RNAs initiated upstream of the rDNA region is also directed by pPolI-CAT, but these RNAs appear less likely to encode enzymatically active CAT protein since there are at least three ATGs (at rDNA positions −125, −100, and −45) prior to the ATG at the start of the CAT-encoding region.

The conclusion that RNA transcripts that initiate at the normal rRNA initiation site do not yield translatable mRNA is further strengthened by results with p-2kbPolI-CAT, a construct that contains the entire rDNA promoter sequence

from −2 kb to +57 but does not direct synthesis of translatable CAT RNA (Fig. 2, lane 3). The production of aberrantly initiated RNAs in cells transfected with plasmids bearing small rDNA promoter regions is also consistent with data of Smale and Tjian (23), who transfected COS cells with hybrid genes containing human rDNA promoter regions that had undergone 5′ deletions. These authors found that genes with large upstream rDNA segments (≥150 bp) initiate principally at −1 of the rDNA sequence, while genes carrying upstream domains with deletions direct production of increasing amounts of aberrantly initiated RNA (in the human case beginning at rDNA position −15 and −20), which are proposed to derive from transcription by RNA polymerase II.

The analogy between the present mouse data and the human data (23) suggests that the RNA initiated at +32 of the mouse rDNA might also be transcribed by RNA polymerase II rather than polymerase I. Several additional lines of evidence support this hypothesis. First, this RNA does not appear to be made in an S-100 extract of mouse cells that initiates transcription by polymerase I and III but not by polymerase II (B.S.-W. and K. Miller, unpublished observations). Second, the level of this species is increased when an MSV enhancer is located in *cis* (Fig. 4*A*, lanes 1 and 2), while RNA initiated correctly at position +1 is not (Fig. 3, lanes 3 and 7). Third, the +32 start occurs at a site 3′ to a TATAA-like sequence. Finally, as we have previously shown (20), in pPolI-CAT-transfected cells treated with actinomycin D for 1 hr the +32 species remains while the +1 species is lost. This difference in stability is most likely due to the presence of a 5′ cap structure on the +32 species, since in previous studies the lack of a 5′ cap structure has been shown to cause dramatically lower RNA stability (1).

Transfection into CHO cells shows that neither pPolI-CAT, pSV0′-CAT, nor their enhancer-containing derivatives direct synthesis of functional CAT mRNAs that produce measurable CAT enzymatic activity (Fig. 5). Moreover, in these cells pPolI-CAT-MSV yields detectable transcripts initiating only at position +1. Thus, our data mandate the unexpected conclusion that gene expression in the CHO cells is considerably more stringent than in the L cells, since sequences that are fortuitously utilized in L cells to direct mRNA production are not utilized in CHO cells.

The mouse rDNA promoter is active in CHO cells as well as in mouse cells (Fig. 4*B*). Thus, the species-specific interactions that discriminate mouse from human rDNA templates (24–26) do not do so between mouse and CHO rDNA. This result [which is consistent with recent data of Dahr *et al.* (27)] reinforces the growing body of evidence suggesting that speciation is not causally coupled to changes in rRNA promoter specificity.

Since the RNA polymerase I-transcribed hybrid RNAs are not able to direct protein synthesis, what then is the fate of these transcripts? Are these RNAs recognized by the enzyme systems that polyadenylylate and splice pre-mRNAs and transport them to the cytoplasm? Unfortunately, strong conclusions concerning these questions cannot be drawn from the present data. As the result of the short-lived nature of the +1-initiated pPolI-CAT RNA, we have not yet demonstrated spliced transcripts initiating at +1. Such problems are exacerbated in mouse cells by the presence of other CAT transcripts, which likely derive from synthesis by polymerase II (see above). We have also failed to detect rRNA initiated at +1 that is polyadenylylated or localized in the cytoplasm (M.A.L., unpublished data), but this may simply reflect the instability of the polymerase I-directed transcripts rather than their unsuitability for the polymerase II processing/transport pathway.

Finally, our experiments address the mechanism of action of RNA polymerase II enhancer sequences. The presence of MSV enhancer sequence on the CAT plasmid that lacks any normal eukaryotic promoters (pSV0′-CAT) markedly raised the level of CAT RNAs that initiated fortuitously (presumably under direction of polymerase II). Similarly, the MSV enhancer also raised the level of RNAs that initiated aberrantly within the rRNA region of pPolI-CAT. In contrast, the level of RNA that was correctly initiated at residue +1 was not increased by an MSV enhancer located in *cis* (Fig. 3, lane 7). Thus, transcription by polymerase I is not stimulated by this polymerase II enhancer segment. Complementary studies (J. Windle and B.S.-W., unpublished observations) have shown that an RNA polymerase II promoter placed in *cis* with a frog polymerase I enhancer segment also fails to enhance the level of transcription. These data therefore suggest that polymerase II and I enhancers act in polymerase-specific manners.

We thank Drs. Steve McKnight and George Khoury for advice and the latter for providing pSV2-CAT and the MSV enhancer. We also thank Ms. Sue Millionie for typing the manuscript. This work was supported by Grant GM 34231 from the National Institutes of Health to B.S.-W. and D.W.C.; D.W.C. is also the recipient of a Research Career Development Award from the National Institutes of Health.

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EXHIBIT 4

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Constitutive Expression of an ISGF2/IRF1 Transgene Leads to Interferon-Independent Activation of Interferon-Inducible Genes and Resistance to Virus Infection

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Interferon (IFN)-stimulated gene factor 2 (ISGF2) plays a role in transcription of the beta IFN (IFN-β) gene and IFN-stimulated genes (ISGs) and may function as a central mediator of cytokine responses. Constitutive ISGF2 transgene expression resulted in substantial resistance to three RNA virus families. This phenotype was not a consequence of IFN production and may have arisen directly through ISG expression. ISGF2 acted generally as a positive transcription factor through binding sites from several genes, in the context of transient cotransfection. Constitutive transcription of the endogenous IFN-β gene, and several genes that are normally induced by either IFN-α or IFN-γ, or only by IFN-α, was elevated in cells that constitutively express an ISGF2 transgene. However, constitutive and virus-induced levels of IFN-β mRNA were unaffected in such cell lines.

Interferons (IFNs) were named for interference with viral infections and are also well known for their effects on cell growth and differentiation. These cytokines are grouped in two classes. Type I IFNs include an alpha interferon (IFN-α) gene family, which is expressed predominantly in leukocytes, plus the IFN-β gene, which is expressed in fibroblasts. Type II IFN is known as IFN-γ and is produced predominantly, if not exclusively, by activated T cells. The exact effect of IFN treatment depends on which cell is exposed to which IFN (reviewed in reference 11). Type I and type II IFNs bind to distinct receptors (1, 28, 63). Several genes activated rapidly solely in response to IFN-α have been called IFN-stimulated genes (ISGs) (29, 31, 53). The 6-16 gene is also in this class (13). Some genes, such as that for guanylate-binding protein (GBP), HLA-A2 (and other class I major histocompatibility complex antigens), and 9-27, are immediately induced by either IFN-α or IFN-γ (3, 8, 9, 14, 35, 55). It is generally accepted that the ability to elicit an antiviral or antiproliferative state is due to transcriptional induction of the ISGs and other previously quiescent genes and the resultant production of new proteins. It has been shown that cells resistant to IFN-α are deficient in ISG activation (26). However, the contribution of individual proteins to the biological effects of IFN are largely unknown.

An IFN-stimulated response element (ISRE) has been shown to be necessary and sufficient for IFN-α induction of ISGs (25, 53). Three ISG factors (ISGFs) found in nuclear extracts bind to the ISRE in vitro. ISGF1 is constitutive (33). ISGF2 is present at a low basal level, and its synthesis is induced in response to type I or II IFN, as well as tumor necrosis factor alpha, interleukin 1-α, and virus infection (21, 33, 45, 47). ISGF2 is also known as IRF-1 and can bind to the PRD-1 regulatory element of the IFN-β gene (47). ISGF3 is composed of a DNA-binding subunit and additional latent subunits (15). Analyses of DNase-hypersensitive sites and footprints in vivo are consistent with the appearance of ISGF DNA-binding activities in extracts and transcription of ISGs measured by the nuclear run-on assay (40, 46). Many lines of evidence show that ISGF3 mediates signal transduction and the immediate transcriptional activation of ISGs (15, 25, 26, 32, 33) in response to IFN-α .

The role of ISGF2 has become a key question with regard to both the transcriptional activation of IFN genes and the attainment of an antiviral or antiproliferative state after cells are exposed to IFNs. Prolonged exposure to IFN is required to produce these biological endpoints (11, 44), which suggests that steps beyond the well-studied immediate transcriptional responses must be involved. ISGF2 was the first transcription factor found to be newly synthesized in response to IFNs and seemed likely to be a critical part of such a pathway. To help unravel the biological function of ISGF2, the protein was purified and cDNA clones were obtained (47). During these studies, it was found that ISGF2 induced by virus infection, IFN-α, or IFN-γ had essentially the same steady-state distribution of phosphorylated isoforms. The low constitutive level of ISGF2 also exhibited similar isoforms. Differences in the function of ISGF2 dependent on the means of induction could still occur, but would reflect other changes effected by its inducers, or very subtle variations in ISGF2 itself. By manipulating the expression of endogenous ISGF2, it has been found that ISGs and the IFN-β gene can be induced in the near absence of ISGF2, and conversely, treatments that produce high levels of ISGF2 do not necessarily induce these genes (47). However, transcription of these genes can be induced in the presence of high levels of ISGF2. Thus, ISGF2 does not seem to be a transcriptional repressor. Cotransfection studies have shown that in mouse L cells, ISGF2 can activate transcription from a synthetic multimerized binding site strongly, but the native IFN-β promoter only weakly (20). Yet in mouse P19 EC cells, native IFN and *H-2L*^{d} promoters are good targets in a cotransfection assay (21). Perhaps ISGF2 does contribute to IFN-β or ISG transcription and thus help mediate induction of or biological responses to type I IFN, when present in the right context. The ambiguities inherent in conclusions dependent on cotransfection experiments and manipulations with agents that have pleiotropic effects have required additional studies.

In this report, it is shown that cotransfection of an ISGF2 expression construct leads to activation of reporter genes via authentic binding sites from the IFN-β, GBP, or ISG15 promoters. To validate and extend these results, I made

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stable cell lines to obtain elevated constitutive expression of an ISGF2 transgene. The endogenous IFN-β gene and endogenous genes that contain an ISRE, including HLA-A2, GBP, and ISG15, were transcribed at an elevated basal rate in cells that expressed the ISGF2 transgene compared with control cells that did not. However, with a polymerase chain reaction (PCR)-based assay, constitutive IFN-β mRNA was not detected in any of the cells, and no differences between experimental and control cell lines were seen in virus induction of IFN-β mRNA. In addition to this first direct demonstration of transcriptional effects on endogenous genes, these studies also uniquely address the biological role of ISGF2. Cells that expressed the ISGF2 transgene were resistant to infection by picornavirus, paramyxovirus, and rhabdovirus. There was no detectable production of IFN by these cells, and anti-IFN neutralizing antibodies did not change the virus resistance. Among the genes induced by IFN, ISGF2 may have a broad role in the pathway that leads to the antiviral state.

MATERIALS AND METHODS

Plasmids. Reporter constructs contained one or four copies of an ISGF2-binding site plus GATC cohesive ends, ligated and inserted into a *Bam*HI site upstream from a human immunodeficiency virus minimal promoter (57), linked to a chloramphenicol acetyltransferase (CAT)-coding sequence and followed by simian virus 40 splice and polyadenylation sequences, as described previously (36). The HIVCAT plasmid was used as a negative control. The ISGF2-binding sites (in boldface type) were within sequences from IFN-β (GA**GAAGTGAAA**GTGGGAAATT CCT), ISG15 (CTCGGGAAA**GGGAAACCGAAA**CTGAA GCC), and GBP (CCCTAATATG**AAACTGAAA**GTAGT ACTA).

An epidermal growth factor receptor expression construct (38) was modified as follows to make ISGF2 expression constructs. For all constructs, EcoGpt sequence was replaced with an *Eco*RI fragment from pSV_2Neo that includes the neomycin phosphotransferase sequence. SV-ISGF2 was then made by replacing the epidermal growth factor receptor cDNA with ISGF2 cDNA (47). MT-ISGF2 was made by a further substitution of human metallothionein II enhancer and promoter sequences (24) in place of the simian virus 40 control elements that were upstream of the ISGF2 sequence in SV-ISGF2. MT-Xba lacked ISGF2 cDNA sequence, but was otherwise the same as MT-ISGF2. The CMVβ-Gal plasmid was from California Biotechnology, Inc.

Cell culture, transfection, and cytopathic effect assays. HeLa S3 cells (ATCC CCL 2.2) were grown as monolayer cultures in Dulbecco modified Eagle's medium (GIBCO/BRL) plus 10% calf serum (HyClone). DEAE-dextran transfections were performed essentially according to a standard protocol, modified as previously described (36). Each transfection included 20 μg of MT-ISGF2 and/or MT-Xba (combined), 2.5 μg of reporter construct, and 2 μg of CMVβ-Gal internal standard. Cells were treated with 100 μM $ZnSO_4$ approximately 24 h posttransfection. Extracts were made approximately 48 h posttransfection and assayed for β-galactosidase and then for CAT by standard procedures (60).

To obtain stable cell lines, HeLa S3 cells were transfected with SV-ISGF2 as a calcium phosphate precipitate by a standard method (19) and selected for resistance to G418 (GIBCO/BRL). Individual colonies were propagated and maintained under continuous selection with G418 at 250 μg/ml. Expression of functional ISGF2 was determined (see below) to evaluate the cell lines.

For cytopathic effect assays, viruses were obtained from the following individuals: encephalomyocarditis virus (EMCV) from Jan Vilcek, Newcastle disease virus (NDV) from Pravin Sehgal, and vesicular stomatitis virus (VSV) (Indiana serotype) from Lawrence Pfeffer. Monolayer cultures in 96-well plates were near confluence when infected with EMCV or VSV or approximately 30% confluent when infected with NDV. Viruses were diluted in medium without serum and added directly to the culture medium in each well. The final serum concentration was at least 5%. Approximately 24 h after EMCV or VSV infection, or approximately 72 h after NDV infection, the monolayers were stained as described previously (37).

Whole-cell extracts and electrophoretic mobility shift assay. All steps for extract preparation were done at 0 to 4°C. Monolayer cultures in 24-well plates were washed with phosphate-buffered saline and then scraped with a rubber policeman into 50 μl of extraction buffer (0.5% Nonidet P-40, 0.3 M NaCl, 0.1 mM EDTA, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 10% glycerol; 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 3 μg of aprotinin per ml, 1 μg of leupeptin per ml, and 2 μg of pepstatin per ml were freshly added to the buffer before each use). The samples were transferred to microcentrifuge tubes, incubated for 60 min with occasional mixing, and then clarified by centrifugation for 5 min at 13,000 × *g*. The supernatants were recovered and assayed for DNA-binding proteins with an oligonucleotide probe that contained the ISG15 ISRE sequence as previously described (47).

Determination of transcription rates. Run-on assays were performed with isolated nuclei to determine relative rates of transcription as previously described (29). Fragments containing HLA-A2 genomic sequence (27), ISGF2 cDNA (47), ISG15 or ISG54 exons (31, 53), IFN-β cDNA (gift of E. Knight, DuPont), GBP cDNA (6), or a β-tubulin pseudogene (66) were isolated from previously described constructs (6, 48) and used as probes. M13mp18 replicative-form DNA was used as a negative control probe.

RNA preparation and PCR assay. Total cytoplasmic RNA was prepared from monolayer cultures in 6-well plates 12 h after mock infection or VSV infection (multiplicity of infection [MOI] = 1), according to a standard Nonidet P-40 lysis protocol (60). Equal aliquots of these RNA samples, mixed with total cytoplasmic RNA from rat 01 cells (constitutive IFN-β producers [42]), or of the rat RNA alone were treated with DNase. The digestion was stopped by phenol extraction, and the RNA was recovered by ethanol precipitation. The recovered RNA was primed with oligo(dT) and incubated in the absence or presence of reverse transcriptase. These samples were then used for PCR. IFN-β sequences were amplified as described previously (42). The reaction products were electrophoresed and blotted to Zetaprobe (Bio-Rad). The same primers and reaction conditions as used for the RNA samples were used to amplify rat or human genomic IFN-β sequences; then the amplified fragments were recovered, radiolabeled with [α-^{32}P]dATP by random priming (12), and used to probe the PCR-amplified RNA and control samples.

RESULTS

General role for ISGF2 as a positive transcription factor. Differences in the outcome of cotransfection experiments

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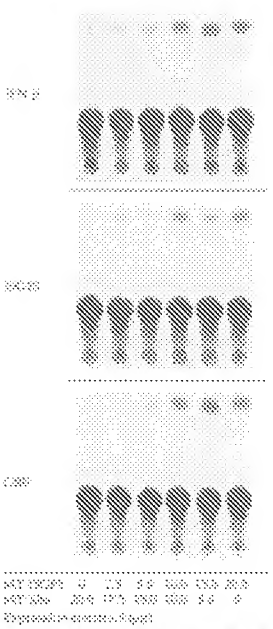

FIG. 1. ISGF2 can activate expression from various authentic binding sites. The amount of the MT-ISGF2 expression construct for each transfection increased from 0 to 20 μg, but the total DNA was kept constant by corresponding decreases in the amount of the control MT-Xba vector from 20 to 0 μg, as indicated. Each transfection also included fixed amounts of a CMVβ-Gal plasmid as an internal standard and the indicated reporter construct. The reporter constructs contained four copies of the ISG15, GBP, or IFN-β promoter sequences shown in the experimental procedures, fused to the CAT gene. Extracts normalized for β-galactosidase activity were assayed for CAT expression from the reporter constructs. The ISG15 construct resulted in very high basal activity and correspondingly higher induced activity. Therefore, CAT assays were done with 1/10th as much of these extracts as of the others, on the basis of β-galactosidase activity.

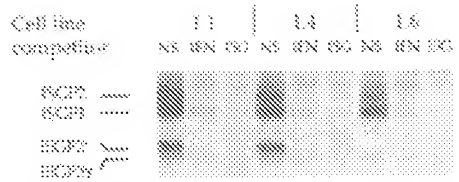

FIG. 2. Constitutive expression of an ISGF2 transgene in stable cell lines. HeLa S3 cells were transfected with an expression vector that contained transcription units to express ISGF2 cDNA and neomycin phosphotransferase, each under the control of a separate simian virus 40 enhancer and promoter element. Cell lines were expanded from single G418-resistant colonies. A one-step procedure was used to prepare crude whole-cell extracts, which were assayed by electrophoretic mobility shift with an ISG15 ISRE oligonucleotide probe. Reaction mixtures contained unlabeled oligonucleotides of sequences from an unrelated gene as nonspecific competitor (NS), from the IFN-β promoter to compete against ISGF1, ISGF2, and closely related proteins (IFN), or the ISG15 ISRE, which was also used as the probe, to compete against all specifically bound proteins (ISG). The specific DNA-protein complexes are as indicated. The excess free probe is not shown.

with synthetic ISGF2-binding sites compared with native IFN-β promoter sequences as the target in mouse L929 cells (20) and discrepancies between such results and studies of endogenous IFN-β gene transcription in HeLa cells (47) require further clarification of the activity of this factor. The possibility that ISGF2 activates other genes, such as ISGs, that contain a binding site is also unresolved. To test that possibility and investigate the generality of any role for ISGF2 in the activation of the IFN-β gene, transient cotransfection experiments were performed with HeLa S3 cells. Reporter constructs were made with one or four copies of the PRD-1 and the PRD-2 elements from the human IFN-β promoter, the ISRE sequence from ISG15, or the ISRE sequence from the GBP gene, upstream of a human immunodeficiency virus minimal promoter fused to CAT-coding sequence, and then splice and polyadenylation sequences from simian virus 40. It has previously been shown that binding of ISGF2 to these sites is based on the presence of a nonamer consensus sequence (25, 47) and that the PRD-2 element in the sequence from the IFN-β promoter is a binding site for NF-κB or related factors, but not ISGF2 (16, 22, 30, 47, 64).

Each of these plasmids was transfected into HeLa S3 cells with a mixture of an ISGF2 expression vector (MT-ISGF2) and the same vector lacking the ISGF2 cDNA insert (MT-Xba). As the amount of the expression vector was raised from 0 to 20 μg, and the control vector was correspondingly lowered to maintain a constant total amount of DNA, each reporter construct was increasingly activated (Fig. 1). The assay after transfection with 20 μg of MT-Xba produced 0.5, 0.8, and 0.6% acetylated chloramphenicol and maximal activation with 20 μg of MT-ISGF2 yielded 1.8, 1.3, and 1.5% acetylated chloramphenicol for the cotransfected IFN-β, ISG, and GBP constructs, respectively. Similar results were obtained in three additional experiments with these reporter constructs and with reporter constructs that contained a single copy of a binding site (data not shown). The very low basal activity of the minimal HIVCAT reporter, with no ISGF2-binding site inserted, was the same whether cotransfected with 20 μg of MT-Xba or 20 μg of MT-ISGF2 (data not shown). Thus, in HeLa S3 cells, ISGF2 can activate transcription from the IFN-β or ISG regulatory elements in the context of a transient transfection, although weakly. It is unlikely that activation of the ISRE-containing reporters is due to production of IFN by the transfected cells (see below).

Molecular effects of constitutive ISGF2 transgene expression in stable cell lines. Examination of stable cell lines that include an ISGF2 transgene was undertaken to distinguish between what can happen in a cotransfection experiment and what does happen to endogenous genes when the ISGF2 transgene is integrated in chromatin and constitutively expressed. G418-resistant colonies were isolated, expanded, and propagated under continuous selection after transfection of HeLa S3 cells with an expression vector that encoded both ISGF2 and neomycin phosphotransferase in separate transcription units. Whole-cell extracts prepared by a single-step procedure were assayed for the level of ISGF2 DNA-binding activity by electrophoretic mobility shift assay with an ISG15 ISRE sequence as the probe.

Figure 2 shows the specific ISGF-DNA complexes in assays of three G418-resistant cell lines. Cell lines 1.1 and 1.4 have distinctly increased ISGF2 DNA-binding activity. In contrast, cell line 1.6 exhibits a typical low constitutive level of ISGF2, which is much lower than the constitutive ISGF1-binding activity, as is the case for the parental HeLa

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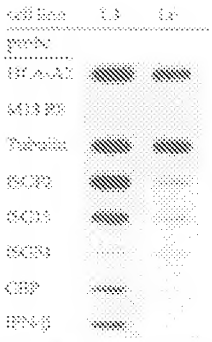

FIG. 3. Constitutive transcription of IFN-stimulated and IFN-β genes is higher in the ISGF2 transgene-expressing cell line 1.4 than in the control cell line 1.6. Nuclei were isolated from the 1.4 and 1.6 cell lines, and nascent RNA was elongated in the presence of [α-^{32}P]UTP. Radiolabeled RNA was isolated and hybridized to excess DNA fixed to nitrocellulose to determine the transcription rate of the indicated genes. Tubulin serves as an internal standard, and M13 replicative-form (RF) DNA is a negative control for specificity of hybridization.

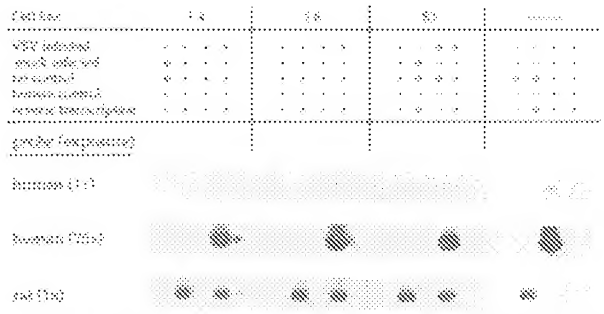

FIG. 4. Constitutive expression of an ISGF2 transgene does not alter the constitutive or virus-induced level of IFN-β mRNA. Total cytoplasmic RNA was prepared from mock-infected or VSV-infected cell lines or HeLa S3 cells as indicated. Each sample was spiked with total cytoplasmic RNA from rat 0l cells, which have a high constitutive level of IFN-β mRNA (42), treated with DNase, and then prepared for PCRs by priming with oligo(dT) and incubating without or with reverse transcriptase as indicated. Control samples included only RNA from the rat 0l cells or human genomic DNA. PCR was performed with primers common to the human and rat IFN-β genes (42). After electrophoresis and blotting, the PCR products were hybridized sequentially with probes specific for the human or rat IFN-β genes. The rat RNA served as an external standard and showed that the efficiency of the amplification was the same in each sample. The assay did measure RNA, since amplification did not occur if reverse transcriptase was omitted, and the probes used did specifically detect either human or rat IFN-β products as demonstrated by control reactions that contained only one or the other template.

S3 cells (for example, see Fig. 8 in reference 47). While there is some variability among the cell lines in the apparent amounts of ISGF1 or ISGF2 from one set of extracts to another, in every case, 1.6 has less ISGF2 than it has ISGF1, while 1.1 and 1.4 always have relatively more ISGF2 than ISGF1. The constitutive level of ISGF2 in cell lines 1.1 and 1.4 is comparable to the level that is induced by IFN-α in cell line 1.6 or parental cells (data not shown). ISGF2 was identified in these extracts by its characteristic mobility, specificity of binding, and reaction with anti-ISGF2 antiserum in the electrophoretic mobility shift assay and on Western blots (immunoblots) (data not shown). A protein labeled as ISGF2′ was present only in cells that also contain an elevated level of ISGF2. ISGF2′ formed a unique protein-DNA complex that had the same binding site specificity as ISGF2. A corresponding new band with apparent molecular mass of 46 kDa was detected on a Western blot with anti-ISGF2 antiserum, and its reactivity with the antiserum was comparable to that of ISGF2 in relation to the level of DNA-binding activity (data not shown). Thus, ISGF2′ is almost certainly also a product of the expression vector. Cell lines 1.1 and 1.4 were used to investigate the consequences of increased constitutive ISGF2′ and ISGF2 expression, while cell line 1.6 was maintained in parallel to serve as a control, along with the parental HeLa S3 cell line.

The transcription rates of several genes that are known to have regulatory elements to which ISGF2 can bind, of ISGF2 itself, and of β-tubulin as an internal standard were directly measured in the experimental and control cell lines, 1.4 and 1.6, respectively. The results of such a run-on assay are shown in Fig. 3. The probe for ISGF2 measures the sum of transcription rates of both the endogenous gene and the transgene, which is greatly increased in cell line 1.4 compared with 1.6, consistent with the increased constitutive level of ISGF2 protein(s) in those cells. The major histocompatibility complex class I gene HLA-A2 and the GBP gene, both inducible by either type I or II IFNs, the IFN-α-stimulated gene ISG15, and the IFN-β gene all exhibit elevated basal transcription rates in cell line 1.4. The basal transcription rate of the IFN-α-stimulated gene ISG54 is essentially the same in the two cell lines, and the transcription rates of all these genes relative to the tubulin standard are comparable in cell line 1.6 and the parental HeLa S3 cells (47, 54).

To assess the influence of ISGF2 transgene expression on the viral induction of IFN-β gene expression, I measured IFN-β mRNA in mock-infected and VSV-infected experimental, control, and parental cells (Fig. 4). Surprisingly, the constitutive level of IFN-β mRNA in mock-infected cells was essentially undetectable in this PCR-based assay. IFN-β mRNA was induced by VSV infection. However, the level of the induction was comparable in all these cell lines. Cell line 1.1, which is similar to cell line 1.4 in expression of the ISGF2 transgene, also had undetectable constitutive and comparable induced levels of IFN-β mRNA (data not shown). This qualitative comparison of constitutive or induced IFN-β mRNA levels among different cell lines does not depend on a quantitative assay. Detection of the virally induced human IFN-β mRNA provides a positive control for the relative sensitivity of this PCR protocol, even though a constitutive amount of template RNA below the detection limit is inherently below the linear range. However, the strong signal from the rat RNA added as an external standard does show that the level of human IFN-β mRNA detected in samples from virus-infected cells is far from exceeding the capacity of the assay.

Constitutive expression of the ISGF2 transgene confers an antiviral state. Figure 5 compares cell lines 1.4 and 1.6 for the cytopathic effect of VSV infection. As cells were infected with increased amounts of virus, the proportion of cells that are viable 24 h later decreased, as evidenced by the failure to stain with a vital dye. On the basis of the MOI required to achieve near 100% cell death, cell line 1.4 was approximately 30-fold more resistant to infection than the control cell line

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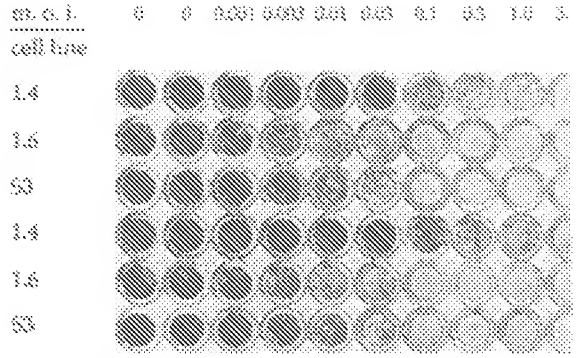

FIG. 5. Cell line 1.4 is resistant to VSV infection. Confluent monolayers of cell line 1.4 or 1.6 or parental HeLa S3 cells were mock infected or infected with increasing multiplicities of VSV as indicated and subsequently stained to reveal the extent of the cytopathic effect, as described in the experimental procedures.

1.6 or the parental HeLa S3 cells. Cell line 1.1 was similarly resistant to the cytopathic effect of VSV infection (data not shown). This significant biological response to constitutive expression of the ISGF2 transgene was a surprise, since little or no IFN-β mRNA had been detected in the resistant cell lines.

Another infection was performed in which cell line 1.4 was compared with cell line 1.6 treated with increasing amounts of IFN-α. The top panel of Fig. 6 establishes cell line 1.6 as a reference for the effect of IFN, and the bottom panel shows the effect of conditioned medium from the experimental cell line on the same reference cells. Cell line 1.4 had resistance to VSV infection comparable to what was conferred on cell line 1.6 by IFN-α added at 100 U/ml. The medium was removed from the wells of the plate shown in the top panel after 24 h of conditioning, before the virus infection, and added to the respective wells of a second plate, shown in the bottom panel, that contained only 1.6 cells. This experiment tested the possibility that the antiviral state of cell line 1.4 was due to production of IFN-α, if not IFN-β. However, the conditioned medium from untreated cell line 1.4 conferred no protective effect, as seen by comparison with the adjacent column, which contained medium conditioned by cell line 1.6, or with the untreated 1.6 cells seen in the top panel. In contrast, the medium to which IFN had been added during conditioning conferred virus resistance to the cells on the second plate comparable to what had been observed for the first plate. Thus, IFN-α in the medium was effective under these circumstances. Clearly, cell line 1.4 did not secrete enough IFN in 24 h to account for its degree of resistance to VSV infection, nor even enough to produce the effect of conditioned medium that had been supplemented with 10 U of IFN-α per ml at the start of conditioning.

Figure 7 shows that the resistance of cell line 1.4 was also not due to chronic low-level production of IFN, which might have produced an antiviral state even though the level of IFN production was below 10 U/ml/day. The leftmost two columns demonstrate that a mixture of neutralizing antisera against IFN-α and IFN-β had no effect on the resistance of cell line 1.4 to VSV infection. The three adjacent columns show that these sera did block the cumulative effect of daily doses of IFN at 10 U/ml, which otherwise provided significant protection against VSV infection. The rightmost column shows that, as expected, a preexisting IFN-induced

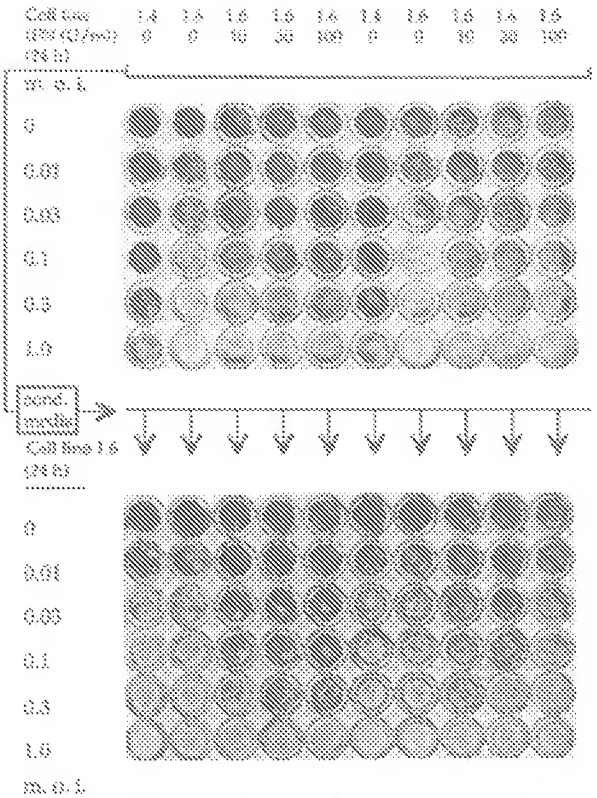

FIG. 6. Cell line 1.4 has virus resistance that is comparable to that of IFN-treated control cells, but does not secrete a corresponding amount of IFN. (Top) Cell lines were seeded near confluence in 96-well plates with medium that contained IFN-α as indicated. After 24 h, the medium was removed, fresh medium was added, and the cells were infected with VSV at the MOI shown. (Bottom) The conditioned medium from the first plate was used to replace the medium from a second plate that contained confluent monolayers of cell line 1.6 in each well. The second plate was infected with VSV as indicated 24 h after the addition of the conditioned medium. The cells on each plate were stained approximately 24 h postinfection.

antiviral state would have decayed during the experiment. Four days after treatment of the control cell line 1.6 for 24 h with 30 U of IFN-α per ml, the cytopathic effect assay showed no difference from untreated control cells.

It still remained possible that the elevated constitutive level of ISGF2 primed cell line 1.4 for rapid and enhanced production of IFN during a virus infection, such that a paracrine protection of the cell population occurred. However, Fig. 8 shows that the presence of the neutralizing antisera during virus infection had essentially no effect on the cytopathic effect of VSV for the experimental resistant, sensitive control, or parental cells. Figure 8 also shows that the resistance of cell line 1.4 to virus infection was a general phenomenon. As seen for the rhabdovirus VSV, attainment of cytopathic effect comparable to what is seen for 1.6 or S3 cells again requires 30- to 100-fold-higher MOI for EMCV, a picornavirus, and for NDV, a paramyxovirus. Cell line 1.1 also had some resistance to these additional viruses (data not shown).

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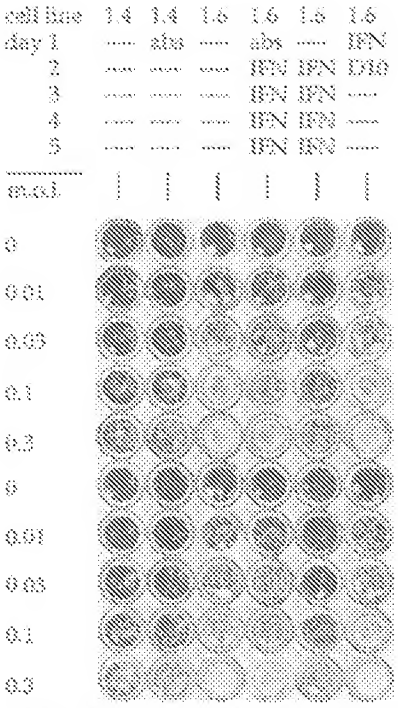

FIG. 7. Antiviral state of cell line 1.4 is unaffected by growth in the presence of neutralizing anti-IFN antibodies (abs). Cell lines were seeded at 5 to 10% confluence in 96-well plates on day 1, and after attachment, 15 U of each IFN per ml or antibodies against IFN-α and IFN-β sufficient to neutralize >500 U of each IFN per ml were added as indicated. On day 2, medium and IFN were removed from the IFN-treated cells and replaced with fresh medium without IFN. Other cells received 5 U of each IFN per ml on each of days 2 to 5 as indicated. Medium was removed from all wells on day 6, and the cells were infected with VSV in fresh medium at the indicated MOI and then stained approximately 24 h postinfection.

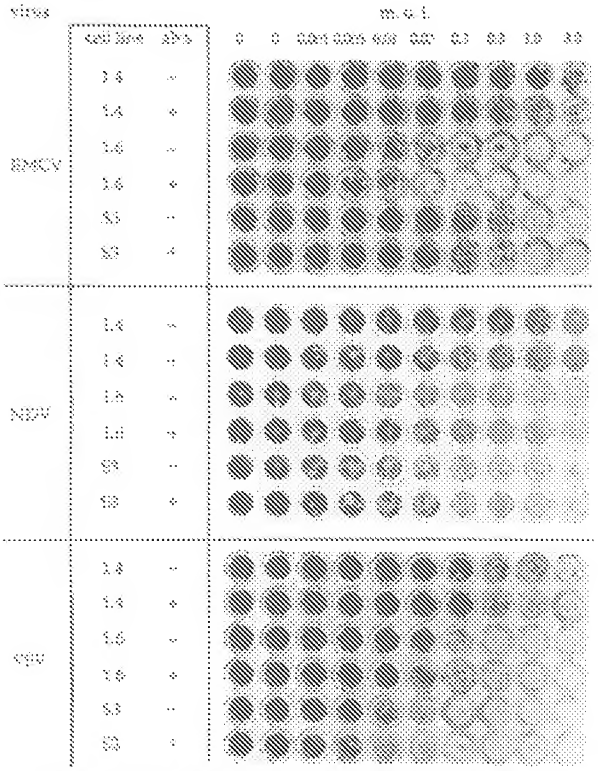

FIG. 8. Cell line 1.4 resistance to three virus families is not a consequence of infection-induced production of IFN. Cell lines or parental HeLa S3 cells were seeded in 96-well plates at 100% confluence (for EMCV or VSV infection) or 30% confluence (for NDV infection) as indicated. After attachment, they were infected, in the absence (−) or presence (+) of antibodies (ab's) against IFN-α and IFN-β sufficient to neutralize >500 U of each IFN per ml, with EMCV, NDV or VSV at the MOI shown. Approximately 24 h (EMCV and VSV) or 72 h (NDV) postinfection, the cells were stained to determine cytopathic effect.

DISCUSSION

The results reported here show virus resistance in cells that stably express an ISGF2 transgene but do not produce any IFN. EMCV, NDV, and VSV, RNA viruses from three different families, were all less able to cause a cytopathic effect in such cells than in the parental cell line. Binding sites from IFN-β, ISG15, and GBP promoters provide targets for activation by cotransfection of an ISGF2 expression vector, and transcription of the corresponding endogenous genes is elevated in the virus-resistant stable cell line 1.4 compared with the control stable cell line 1.6, which is sensitive to virus infection.

Biological consequences of constitutive ISGF2 transgene expression. The biology of cells that stably expressed an ISGF2 transgene was a critical focus of these studies. Two independently isolated clonal cell lines (1.1 and 1.4) that express the ISGF2 transgene were more resistant to EMCV and VSV than the control or parental cells. Cell line 1.4 was also resistant to NDV. Cell line 1.1 was slightly, if at all, more resistant to NDV than cell line 1.6, and both were 3- to 10-fold more resistant to NDV than HeLa S3 cells. The control cell line 1.6 is overall a representative sensitive clone, since it was generally more sensitive than cell lines 1.1 or 1.4 and similar to the parental HeLa S3 cells. Together, these points show that the resistance is not due to clonal variation within the original population of parental cells, but rather can be attributed to expression of the ISGF2 transgene.

The viral resistance was not due to secretion of IFN. Medium conditioned by cell line 1.4 contained less than 10 U of IFN per ml. The conditioned medium conferred no detectable resistance on sensitive cells, but sensitive cells were protected by addition of 10 U of IFN per ml. Neutralizing antibodies against IFN, added before infection, did not diminish the resistance of cell line 1.4 but were able to block the effect of added IFN. Addition of these neutralizing antisera at the start of infection caused little or no increased sensitivity to the cytopathic effect of the viruses tested on any of the cell lines examined. The failure to secrete IFN indicates that induced ISGF2 gene expression alone is not sufficient for biologically relevant up-regulation of IFN-β gene expression in HeLa cells.

Of particular significance was the generality of the resistance. The cells that expressed the ISGF2 transgene were resistant to RNA viruses from three families. Constitutive expression of other individual IFN-inducible genes does not

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give a general effect. Stable cells that overexpress 2′-5′ oligoadenylate synthetase (OAS) are protected against only EMCV (5, 59). Overexpression of murine Mx1, first defined as a gene that renders mice resistant to influenza virus, did make cells influenza virus resistant (2, 62). Resistance to influenza virus, as well as unexpected resistance to VSV, was also observed in cells that express rat Mx1 or human MxA (39, 43). Curiously, rat Mx2 made cells resistant to VSV but not influenza virus. However, the cells made to overexpress these proteins were not resistant to picornavirus. Furthermore, rat Mx3 and human MxB did not provide protection against any virus tested. Thus, overexpression of these gene products confers resistance to one or two virus families, while constitutive expression of the ISGF2 transgene more broadly recapitulates the antiviral effect of IFN treatment and does so even though the resistant cells do not produce IFN.

Significance of ISGF2 activity assayed by transient transfections. Previous studies have focused on the characterization and function of ISGF2 at the molecular level (18, 20, 21, 33, 47), with the aim of elucidating the biological role of this transcription factor. The transient assays described in this report demonstrate that cotransfection of an ISGF2 expression vector can transactivate a reporter construct that contains multiple copies of authentic regulatory elements from the IFN-β gene. This outcome provides evidence for an inherent ability of ISGF2 to positively regulate the IFN-β promoter, at least in the context of a transient transfection. Additionally, the generality of a role for ISGF2 as a positive transcription factor is demonstrated by activation of reporters that present an ISGF2-binding site within the context of the ISRE from ISG15 or GBP, and the ability to activate in a cell type, HeLa S3, other than that previously reported (20, 21). The limited transactivation of these sites in the cotransfection experiments is not a surprise. These promoter elements in endogenous genes are constitutively almost silent. Weak activation of the IFN-β promoter in mouse L cells compared with better activation in EC cells was explained in terms of differences in constitutive negative regulation (21). However, it also seems likely that, by itself, ISGF2 is not a strong transcriptional activator. Thus, it is imperative that cotransfection data be taken as indicative of, rather than proof for, activity of endogenous ISGF2 on particular endogenous genes. In addition to the general conclusions that such experiments do support, they dictate a need and provide a guide for the design of direct experiments to validate specific hypotheses suggested by the transient cotransfections.

Molecular consequences of constitutive ISGF2 transgene expression. A suggestion that ISGF2 plays an important role in virus induction of IFN-β gene transcription has been based on the kinetics of ISGF2 and IFNβ mRNA accumulation, activation of cotransfected reporters, and in vitro binding assays (20, 21, 41, 65), but was not supported by any direct transcriptional analysis of endogenous genes. This report provides a direct assay that demonstrates increased transcription of the endogenous IFN-β gene in cells that stably express the ISGF2 transgene. Since the increased transcription of the IFN-β gene in cell line 1.4 does not lead to constitutive accumulation or influence viral induction of the mRNA, posttranscriptional mechanisms must help regulate the level. In human fibroblasts, IFN-β mRNA is known to be targeted for rapid turnover, and NDV infection increases its half-life (51, 52). A specific sequence conserved in the 3′-untranslated region of IFN and many other inflammatory mediator mRNAs (4) has been shown to direct selective degradation of granulocyte-macrophage colony-stimulating factor mRNA (61). Thus, it seems that ISGF2-induced IFN-β gene transcription alone cannot mimic the effect of virus infection, and stabilization of IFN-β mRNA that occurs during infection is essential for accumulation. The fact that VSV induction of IFN-β mRNA in the stable cells that express the ISGF2 transgene is unaffected suggests that the increased constitutive IFN-β gene transcription in those cells is relatively minor compared with the combined affects on transcription and mRNA stability that are the normal HeLa cell response to virus infection.

With a different induction regimen or in a different cell type, elevated constitutive ISGF2 gene expression and resultant induced IFN-β gene transcription might influence constitutive or virally induced levels of IFN-β mRNA. In fact, a report that describes the influence of constitutive sense or antisense expression from an ISGF2 transgene in transformed fibroblasts on NDV or double-stranded RNA induction of IFN-β mRNA accumulation was published while this manuscript was under review (56). No effect on the constitutive mRNA level was observed, consistent with the results presented here, while IFN-β mRNA induction was either enhanced or inhibited in the cells that expressed the sense or antisense constructs, respectively. Curiously, IFN induction of HLA-B7 mRNA was enhanced in the cell lines with the sense ISGF2 constructs, but unaffected by expression of the antisense ISGF2 transgene. However, it was not shown whether any of these results reflected changes in transcription of these genes, or represented an unexpected influence of ISGF2 on posttranscriptional events, in the system studied.

The results from the transcriptional analysis provide a likely explanation for an antiviral state in the absence of IFN secretion by the resistant cells. The broad antiviral activity of IFN must reflect its ability to induce many genes, each of which would confer resistance to a very limited number of viruses, as discussed above for Mx and OAS. Not only OAS but also double-stranded RNA-dependent protein kinase has been implicated in translational control mechanisms that may effect IFN action (48). It will be of interest to determine whether the expression of these particular genes is increased in the virus-resistant cell lines that express the ISGF2 transgene, or whether other gene products must account for resistance to EMCV and VSV. These genes, and many others characterized for the regulatory elements that confer rapid transcriptional induction in response to type I IFNs, have an ISRE (7, 23, 33, 50, 53, 58). Elevated transcription of the major histocompatibility complex class I gene HLA-A2 in cell line 1.4 may implicate ISGF2 in the immunomodulatory effects of IFNs. Such effects augment the direct actions of IFNs on individual cells through an influence on cell-cell interactions that are part of overall host antiviral defenses. Particularly, increased major histocompatibility class expression may enhance immune surveillance of infected cells. The broad virus resistance of cell line 1.4 probably reflects the elevated transcription in cell line 1.4 of many endogenous genes that are normally induced by IFN-α or IFN-γ.

There are clearly additional complexities in the biological role of ISGF2. Although IFN-γ is a far more potent inducer of ISGF2 than IFN-α or IFN-β, and ISGF2 is also induced by interleukin 1α or tumor necrosis factor alpha (17, 45, 47), there is no IFN-γ- or IFN-α-induced transcription of IFN-β (47), and only IFN-α or IFN-β induces transcription of ISGs (29, 45, 47). Additionally, a combination of cycloheximide and double-stranded RNA strongly induces transcription of the IFN-β gene in HeLa cells, despite the near absence of

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ISGF2 (47). There is no contradiction between the data presented here and the strong evidence that ISGF3 mediates signal transduction in response to IFN-α and is responsible for rapid transcriptional induction of ISGs (15, 25, 26, 32–34). The relative contribution of the ISGF2 and ISGF2′ products of the transgene to the transcriptional activation and virus resistance described above is not clear and highlights the need for further structural and functional studies of this factor. Altogether, it is likely that ISGF2 positively modulates the transcription of IFN-induced genes essential to the establishment of a broad-spectrum antiviral state, but not under all circumstances. The contextual differences could include subtle variations in ISGF2 itself, or other variations in the intracellular environment that reflect pleiotropic effects of the agents that induce ISGF2. The transcriptional analysis reported here extends the data from cotransfection experiments (21) and in vitro binding assays (49) to provide new support for the hypothesis that IFN-induced expression of the ISGF2 gene does contribute to regulation of IFN-inducible genes. This hypothesis can be directly examined in future studies with these cell lines.

It seems likely that some genes will be primarily regulated by ISGF2, since it is synthesized in response to many different cytokines and may thus have a central role in cytokine networks. However, all the genes so far tested for regulation by ISGF2 are also, if not predominantly, regulated by other transcription factors, such as ISGF3, GAF, and NF-κB (10, 16, 22, 26, 30, 32, 33, 64). Thus, ISGF2 is an ancillary factor in the transcriptional regulation of these genes. Part of the antiviral state of cell line 1.4 could reflect activity of ISGF2 as an intermediate required for the activation of certain other genes in the overall cellular response to IFN. The availability of these cells will facilitate the identification of such genes.

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EXHIBIT 5

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Biochemistry

Eukaryotic transient expression system dependent on transcription factors and regulatory DNA sequences of vaccinia virus

(transfection/chimeric gene/chloramphenicol acetyltransferase)

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ABSTRACT **A transient expression system in which chimeric genes are expressed in cells infected with vaccinia virus was developed. Recombinant plasmids containing the promoter regions of vaccinia virus genes ligated to the coding segment of the prokaryotic chloramphenicol acetyltransferase (CAT) gene were constructed. When the plasmids were introduced into vaccinia virus-infected cells by transfection, the chimeric gene was expressed and significant levels of CAT accumulated. CAT activity was not detected when the same recombinant plasmid was introduced into uninfected cells, nor was activity detected when the vaccinia virus promoter was absent from the plasmid or was replaced by simian virus 40 or Rous sarcoma virus promoters. This specificity indicated that expression is dependent on a *cis*-acting vaccinia virus promoter region within the recombinant plasmid and diffusible *trans*-acting transcription factors produced during virus infection. The lack of effect of a simian virus 40 enhancer element inserted upstream of the vaccinia virus promoter region also distinguished this system from systems dependent on RNA polymerase II. Although replication of the recombinant plasmid could not be detected in either uninfected or vaccinia virus-infected cells, an inhibitor of DNA synthesis significantly reduced CAT expression. This result, as well as the kinetics of CAT synthesis, suggests that replication of viral DNA templates can enhance transcription of chimeric genes in recombinant plasmids.**

Recombinant DNA that is introduced into eukaryotic cells by transfection or microinjection may be transiently expressed in an unintegrated state (1–11). In such systems, transcription occurs via the RNA polymerase II system within the cell nucleus and is dependent on appropriate *cis*-regulatory sequences associated with the transfected genes. The transcripts must then be properly processed and transported to the cytoplasm for translation to occur. We considered that an analogous cytoplasmic transient expression system might be developed with vaccinia virus-infected cells. Poxviruses, of which vaccinia virus is the prototype, have large DNA genomes encoding biosynthetic enzymes that enable them to use the cytoplasm as a site of transcription and replication (reviewed in ref. 12). Promoter regions upstream of vaccinia virus genes have been identified by both *in vivo* (13–15) and *in vitro* (16) experiments. These regions are extremely rich in adenine and thymine residues and deviate significantly from established eukaryotic consensus sequences (14, 17–19). In this communication, we demonstrate that a heterologous gene ligated to a vaccinia virus transcriptional regulatory sequence is expressed at high levels after transfection of recombinant plasmids. Expression is dependent on the cells also being infected with vaccinia virus, evidently to provide *trans*-acting transcription factors. This transient expression system complements the previously described use of vaccinia virus as a vector for the expression of inserted genes (13, 15, 20).

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MATERIALS AND METHODS

Recombinant Plasmids. Recombinant plasmids were prepared from pBR328 (21), pUC7, or pUC9 (22) and purified as described by Birnboim and Doly (23). DNA fragments were isolated from agarose gels by electrophoresis onto DEAE-paper (24). Plasmids were constructed as indicated in the text, using standard procedures.

Infection, Transfection, and Chloramphenicol Acetyltransferase (CAT) Assays. CV-1 monkey kidney cells were grown to 70% confluency in 25-cm^2 flasks (approximately 2.5×10^6 cells per flask) and infected with wild-type vaccinia virus strain WR at multiplicities stated in the text or incubated with an equivalent volume of medium. After 30 min at 37°C, calcium phosphate-precipitated DNA was added (25, 26). The DNA precipitates contained recombinant plasmid and calf thymus DNA such that the total was 20 μg per ml of transfection mixture. After 30 min at room temperature, fresh medium prewarmed to 37°C was added. When indicated, the medium was supplemented with cytosine arabinonucleoside (araC) at 40 μg/ml. Cells were harvested at various times after infection and suspended in 0.2 ml of Tris·HCl, pH 7.5. After freezing and thawing three times, the disrupted cells were dispersed by sonication and the suspension was assayed for CAT activity as described (15).

RESULTS

Construction of a Chimeric Gene. Gorman *et al.* (1, 27) demonstrated the utility of the prokaryotic *CAT* gene for transient expression studies. The enzyme assay is rapid, sensitive, and quantitative and there is no detectable background CAT activity in eukaryotic cells. Therefore, we wished to construct a chimeric *CAT* gene containing a vaccinia virus promoter region. The entire *CAT* coding segment, without its endogenous promoter, was previously (15) excised from pBR328 (21) with restriction endonuclease *Taq* I and inserted into the *Acc* I site of pUC7 (22). The resulting plasmid, designated pCAT, contains *Bam*HI sites flanking the CAT gene (Fig. 1). A DNA fragment, extending from about 240 base pairs (bp) before and 35 bp beyond the RNA start site of a vaccinia virus gene encoding an M_r 7500 (7.5-kDa) polypeptide, was excised with restriction enzymes *Hin*cII and *Rsa* I (17) and inserted into *Hin*cII-cleaved pUC9 (22). The *CAT* gene was then introduced into the *Bam*HI site of the latter plasmid and the resulting recombinant was called pCP1 (Fig. 1). Since the first ATG downstream of the

Abbreviations: araC, cytosine arabinonucleoside; bp, base pair(s); CAT, chloramphenicol acetyltransferase; pfu, plaque-forming unit; SV40, simian virus 40.

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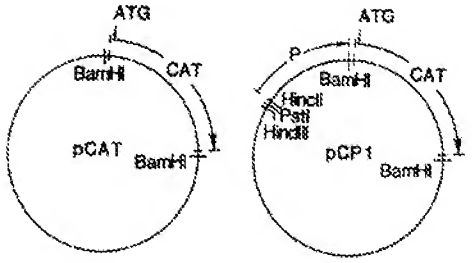

FIG. 1. Structures of recombinant plasmids. The plasmids pCAT and pCP1 contain the entire coding sequence of the *CAT* gene. In pCP1, a DNA fragment including the promoter region of a vaccinia virus gene encoding a 7.5-kDa polypeptide was placed immediately before the *CAT* gene. The arrows indicate the polarity of promoters and coding sequences. The ATG corresponding to a translational initiation codon and relevant restriction endonuclease sites are shown.

viral RNA start site represents the authentic translational initiation signal of the *CAT* gene, expression should result in the formation of active enzyme.

Transient Expression of CAT. To test for expression of the *CAT* gene, pCAT and pCP1 were added as calcium phosphate precipitates to CV-1 monkey cells that were infected or mock-infected with vaccinia virus. The cells were harvested at 24 hr after infection and extracts were tested for CAT activity. As seen in Fig. 2*A*, high levels of CAT were detected in extracts of infected cells transfected with pCP1 but not in extracts of uninfected cells transfected with the same plasmid. Similar results also were obtained with BSC-1 monkey cells and primary chicken embryo fibroblasts (not shown). In contrast, CAT activity was not detected after transfection of infected or uninfected cells with pCAT (Fig. 2*A*). Thus, transient expression appeared to depend on a *cis*-acting vaccinia virus promoter region adjacent to the *CAT* gene and *trans*-acting factors produced during vaccinia virus infection.

The level of transient expression in the vaccinia system was compared to that of a more conventional uninfected cell system. For the latter we used pSV2cat, which contains the simian virus 40 (SV40) enhancer element and early promoter, and pRSVcat, which contains the enhancer and promoter of the long terminal repeat of Rous sarcoma virus (27). We confirmed previous findings of Gorman *et al.* (27) regarding the relative proportions of CAT activity obtained with these two plasmids in uninfected CV-1 cells (Fig. 2*B*). Interestingly, no activity could be measured in extracts from infected cells that had been transfected with pSV2cat or pRSVcat, implying inhibition at some stage needed for expression. Nevertheless, considerable amounts of CAT were made in infected cells that were transfected with pCP1. At 24 hr, the amount of CAT in extracts from infected cells that were transfected with pCP1 was about 40 times higher than that in extracts from uninfected cells that were transfected with pRSVcat.

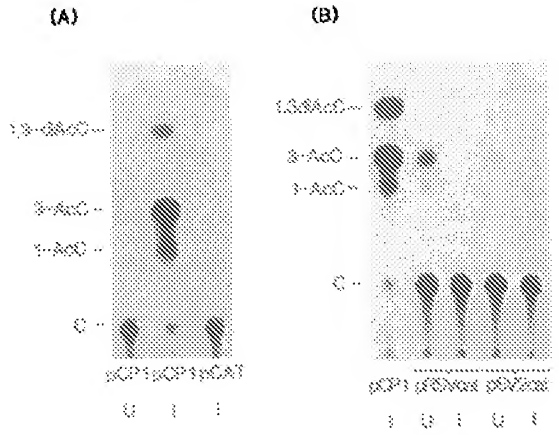

FIG. 2. Transient expression of CAT. (*A*) Uninfected or vaccinia virus-infected CV-1 cells were transfected with 20 μg of pCAT or pCP1 and cell extracts were prepared 24 hr later. CAT assays were performed and analyzed by chromatography on silica gel thin-layer plates. An autoradiograph is shown. (*B*) Uninfected or vaccinia virus-infected CV-1 cells were transfected with 10 μg of pCP1, pRSVcat, or pSV2cat. After 24 hr, extracts were tested for CAT activity as above. U, uninfected; I, infected; C, chloramphenicol; 1-AcC, 3-AcC, and 1,3-diAcC refer to the 1-acetate, 3-acetate and 1,3-diacetate derivatives of chloramphenicol. Autoradiographs are shown.

Since the mechanism of action of eukaryotic transcriptional enhancer elements has not yet been defined, it was of some interest to determine whether such sequences would influence expression of the *CAT* gene under control of a vaccinia virus promoter. The SV40 72-bp repeats have been shown to enhance transcription of CAT and other genes under transient assay conditions (2, 28). Therefore, a fragment containing SV40 DNA (map position 74 to 271) was excised from recombinant plasmid dl74 (29) with restriction enzymes *Sph* I and *Hin*cII and blunt end ligated into the *Sma* I site of pMM23 (19) to form a new plasmid called pMM24. This placed the enhancer-containing SV40 DNA segment in the same orientation upstream of the promoter region of the vaccinia virus 7.5-kDa polypeptide gene as it was relative to the original SV40 gene. Both pMM23 and pMM24 contain the *CAT* coding sequence downstream of the vaccinia virus promoter and are identical except for the presence of the SV40 enhancer in the latter. When vaccinia virus-infected cells were transfected with pMM23 and pMM24, similar levels of CAT expression were obtained, indicating the absence of a detectable enhancer effect.

Optimization of Transient Expression. CAT activity increased in proportion to the amount of helper vaccinia virus added (Fig. 3). This trend leveled off at high multiplicities partly because of increased cell lysis during harvesting, which resulted in measurable amounts of CAT in the medium. Routinely, a multiplicity of 30 pfu per cell was used.

CAT activity also was proportional to the amount of pCP1 used in transfection (Fig. 3). Concentrations of 10–40 μg/ml

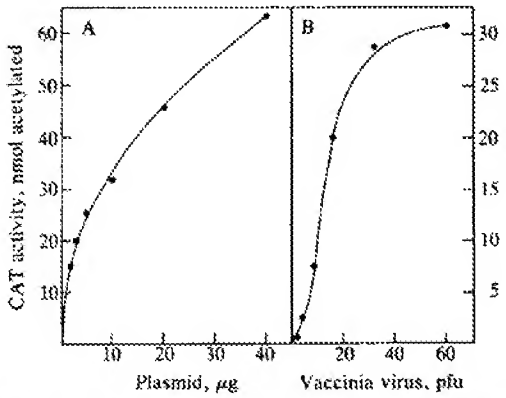

FIG. 3. Dependence of CAT activity on amount of transfected DNA and virus multiplicity. (*A*) CV-1 cells were infected at a multiplicity of 30 plaque-forming units (pfu) per cell and transfected with the indicated amounts of pCP1. (*B*) CV-1 cells were infected with the indicated virus multiplicity and transfected with 10 μg of pCP1. In each case, cells were harvested and analyzed 12 hr after infection. CAT activity is expressed as nmol of chloramphenicol acetylated per 2.5×10^6 cells.

were used for most experiments. The form of the plasmid was critical, since linearization of pCP1 by cleavage at the *Sma* I site, which lies distal to the *CAT* gene (Fig. 1), reduced activity by at least 90%.

The time course of CAT synthesis in cells infected with wild-type vaccinia virus and transfected with pCP1 is shown in Fig. 4. These results are compared to those obtained in cells infected with recombinant vaccinia virus vC24 (15), which contains the *CAT* gene under control of the same promoter used for pCP1. To keep conditions as uniform as possible, cells that were infected with vC24 were transfected with the pUC9 vector, which contains no vaccinia virus sequences. (Calcium phosphate transfection procedures significantly reduced CAT expression by recombinant virus.) CAT was detected within 2 hr after vC24 infection but only 4–6 hr after transfection with pCP1. This lag in transient expression was also observed when the cells were transfected 24 hr before infection with wild-type virus. The levels of CAT synthesized by recombinant virus and in the transient system increased linearly for at least 24 hr. However, the amount of CAT made under the latter conditions was about 30% of that expressed from the recombinant virus.

araC, an inhibitor of DNA replication, typically prevents the expression of late genes of vaccinia virus. However, this drug reduced CAT synthesis in cells infected with vC24 only by about 50% (Fig. 4), in agreement with previous results (15). This partial effect is related to the presence of separately regulated early and late RNA start sites within the promoter region (unpublished data). In the transient system, araC inhibited CAT expression by about 75% (Fig. 4) because of predominant use of the late RNA start site (unpublished data).

The effect of cytosine arabinoside on transient expression could be due to inhibition of virus or pCP1 DNA replication. Two independent methods were used to determine whether replication of pCP1 actually occurs in cells infected with vaccinia virus. DNA was extracted from cells that were uninfected or infected with vaccinia virus in the presence or absence of araC, at various times after transfection with pCP1. Autoradiographs, prepared after immobilization of the DNA on nitrocellulose and hybridization to ^{32}P-labeled pUC9 DNA, revealed no evidence of plasmid replication. The sensitivity of this experiment was increased by using restriction enzymes to distinguish between the input methylated pCP1 grown in bacteria and any unmethylated pCP1 produced by replication in eukaryotic cells (30, 31). DNA was isolated by the Hirt (32) procedure and then digested with *Bam*HI and either *Dpn* I or *Mbo* I. The latter two enzymes recognize the same four-base G-A-T-C sequence, but *Dpn* I cleaves only when the A residue is methylated at the 6-position and *Mbo* II cleaves when it is unmethylated. Since there is a single *Bam*HI site in pCP1 but many G-A-T-C sequences, unmethylated DNA would be linearized by the combination of *Bam*HI and *Dpn* I and digested into many small fragments by the combination of *Bam*HI and *Mbo* I. Conversely, methylated DNA would be linearized by the combination of *Bam*HI and *Mbo* I and digested into small fragments by *Bam*HI and *Dpn* I. Inspection of the overexposed autoradiograph in Fig. 5 reveals no detectable unmethylated pCP1 formed. Since there appears to be no replication of this plasmid under the conditions of transient infection, the effects of araC may be attributed to inhibition of virus DNA replication.

Use of Additional Vaccinia Virus Promoters. Promoter regions from several vaccinia virus genes have been sequenced (14, 17–19, 34), and three of these have been used to express genes inserted into vaccinia virus (13, 15, 19, 35). Use of the promoter region from the 7.5-kDa polypeptide gene has already been described. An additional promoter region was derived from an early gene encoding thymidine kinase and another from a late gene encoding a 28-kDa polypeptide. Recombinant plasmids containing these promoters ligated to the *CAT* gene were tested in a transient assay. As shown in Table 1, CAT expression occurred with the other promoters,

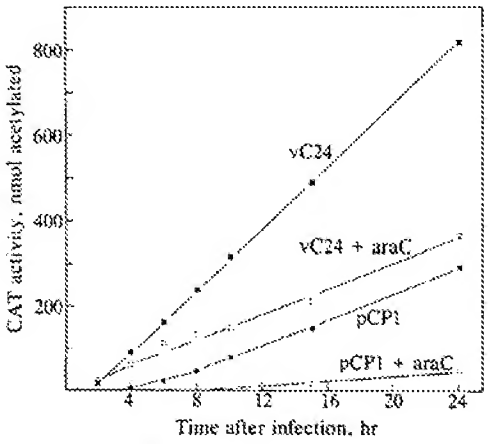

FIG. 4. Time course of CAT synthesis. Replicate cultures of CV-1 cells were infected with wild-type vaccinia virus at 30 pfu per cell and transfected with 20 μg of pCP1 or infected with recombinant virus vC24 at 30 pfu per cell and transfected with pUC9. Cultures were incubated in the absence (filled symbols) or presence (unfilled symbols) of araC at 40 μg/ml. At the indicated times after infection, cells were harvested and assayed for CAT. Activity is expressed as nmol of chloramphenicol acetylated per 2.5×10^6 cells.

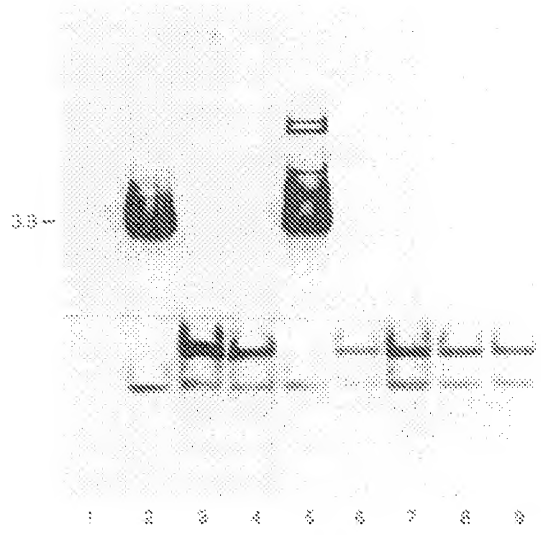

FIG. 5. Absence of pCP1 replication. Vaccinia virus-infected and uninfected CV-1 cells were transfected with 10 μg of pCP1 and maintained in the presence or absence of araC. DNA was prepared 5 and 24 hr after transfection and digested with *Bam*HI and *Dpn* I or *Bam*HI and *Mbo* I. DNA purified from about 5×10^5 cells was analyzed by electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose (33), and hybridized to ^{32}P-labeled pCP1 DNA. An autoradiograph is shown. Migration of the linearized 3.8-kilobase plasmid is indicated. Lane 1, vaccinia virus DNA (100 ng) digested with *Bam*HI and *Dpn* I; lane 2, pCP1 DNA (100 ng grown in a *dam*$^+$ *Escherichia coli* strain) digested with *Bam*HI; lane 3, pCP1 DNA (100 ng grown in a *dam*$^+$ *E. coli* strain) digested with *Bam*HI and *Dpn* I; lane 4, infected, 24 hr, *Bam*HI and *Dpn* I; lane 5, infected, 24 hr, *Bam*HI and *Mbo* I; lane 6, infected, +araC, 24 hr, *Bam*HI and *Dpn* I; lane 7, infected, 5 hr, *Bam*HI and *Dpn* I; lane 8, infected, +araC, 5 hr, *Bam*HI and *Dpn* I; lane 9, uninfected, 24 hr, *Bam*HI and *Dpn* I.

Table 1. Transient activity with different vaccinia virus promoters

Promoter	Plasmid	CAT activity, nmol chloramphenicol acetylated per 2.5×10^6 cells: No drug	With araC
7.5-kDa	pCP1	380	100
Thymidine kinase	pMM19	27	15
28-kDa	pLCAT1	67	14
—	pCAT	<0.01	<0.01

Recombinant plasmids contained the promoter region from the gene encoding 7.5- or 28-kDa polypeptides or thymidine kinase ligated to the *CAT* gene. CV-1 cell monolayers were infected at a multiplicity of 30 pfu per cell and transfected with 20 μg of the indicated plasmid, and cell extracts were prepared 24 hr later.

although at a lower level than with the 7.5-kDa promoter. The relative efficiencies of the 7.5-kDa and thymidine kinase promoters in the transient assay (Table 1) corresponded to those obtained with the same constructs inserted into the vaccinia virus genome (15). In addition, araC inhibited transient expression of the late promoter most and the early promoter least.

DISCUSSION

We have developed a rapid way of expressing chimeric genes in primary and continuous cell lines. The method involves the use of recombinant plasmids containing vaccinia virus promoter regions as *cis*-acting regulatory elements. After insertion of the coding segment of a heterologous gene, such as *CAT*, standard transfection procedures were used to introduce the plasmid into vaccinia virus-infected cells. Promoter regions of three different vaccinia virus genes were used successfully in this system, whereas those from SV40 and Rous sarcoma virus were ineffective. This selectivity is consistent with nucleotide sequence differences between vaccinia virus and other eukaryotic promoter regions (14, 17) as well as functional differences determined by *in vitro* transcription (16). The efficiency of transient expression in the vaccinia system compared favorably with that obtained with the more standard uninfected cell system even when strong promoters and enhancers were provided for the latter. The cytoplasmic site of vaccinia virus transcription may contribute to the high efficiency, since Loyter *et al.* (36) reported that only a small proportion of cells that take up calcium-precipitated DNA also transfer it to the nucleus.

Expression of recombinant plasmids in vaccinia virus-infected cells indicates that the transfected DNA is not excluded from the sites of virus transcription or that viral RNA polymerase and other factors are diffusible. The requirement for diffusible viral proteins may explain the 6-hr delay before significant expression is detected. The delay in CAT synthesis is not due to slow DNA uptake, because it was observed even when the plasmid was added 24 hr before infection. Nor is it due to the nature of the promoter used, since expression occurs within 2 hr when the same chimeric gene is inserted into vaccinia virus. Under the latter conditions, rapid expression evidently occurs because the viral enzymes and DNA are packaged within the core of the virus particle.

The physical nature of the recombinant DNA was important, since expression dropped precipitously when the plasmid was linearized. This is probably not due to lack of DNA uptake, because linearized DNA works well for vaccinia virus marker rescue experiments. A requirement for supercoiled DNA also has been found for other transient expression systems (5). It is important to point out that recombination is unlikely to play a significant role in the vaccinia virus transient expression system for several reasons. First, the promoter fragment used was only a few hundred base pairs long, and much smaller ones serve equally well (unpublished data). Second, transient expression is unaffected by flanking the chimeric gene with vaccinia virus DNA sequences that promote homologous recombination. Third, the recombination frequency even under the latter conditions is less than 0.5%, whereas the rate of transient expression may be as much as 30% of that obtained by infection with recombinant virus.

The reduction in transient expression caused by araC, an inhibitor of DNA replication, was noteworthy. Since replication of the recombinant plasmid could not be detected, the requirement appears to be for viral DNA synthesis. The effect of araC was greatest when a late promoter was used to form the chimeric *CAT* gene and least when an early promoter was used, suggesting that the requirement is for regulatory factors that control late transcription. The most extensive analysis was done with a promoter region that contains early and late RNA start sites. Nuclease S1 mapping studies indicated that both RNA start sites were used for transient expression but that the late one predominated (unpublished data).

This transient expression system has proven to be extremely useful in our laboratory for studying the regulatory signals of vaccinia virus promoter regions. For example, it provides a rapid way of monitoring the effects of *in vitro* mutagenesis. Genes other than *CAT* also have been expressed in this system. When the influenza hemagglutinin was used, the protein was processed correctly and inserted into the plasma membrane, as determined by immunofluorescence. This result indicates that the transient system also may provide a useful way of analyzing effects of mutations on protein function. In many respects the transient system complements the use of vaccinia virus as a vector for insertion of foreign genes (13, 15, 20, 35).

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